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FACULDADE DE CIÊNCIAS
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Molecular epidemiology and antibiotic resistance of C*lostridium difficile*: focus on toxin A-negative/toxin B-positive strains isolated in Portugal

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Mestrado em Microbiologia Aplicada
Dissertação

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2017

Acknowledgements

Conducting a master's thesis implies not only a lot of us but a lot of the others around, therefore, there are contributions of a diverse nature that must be highlighted. First, I would like to thank Dr. Mónica Oleastro, my supervisor at INSARJ. Thank you to receive me at your laboratory, for the scientific competence and follow-up throughout the work, demanding high quality of work and patiently encouraging me to improve my skills. As well as for the relevant criticisms, corrections and suggestions made during the orientation.

I would like to express my gratitude to all those who took part in this journey, to the Masters Andrea Santos and Joana Isidro for all the support, whenever necessary, transmitted knowledge, encouragement, help and friendship, it would not have been possible to carry out this thesis without you two.

I am grateful to Dr. Vítor Borges for all the support with bioinformatic issues, having always been available for any clarification and willing to help.

To the Master Leonor Silveira and Dr. Ângela Pista, thank you for the tireless moral support, for the friendship, strength and affection that you always demonstrated to me.

To the Microbial Development Lab from Instituto de Tecnologia Química e Biológica - António Xavier, Universidade Nova de Lisboa that promptly contributed to this work with the transformation of strains, thanks to all the people involved.

I thank all my family for their unconditional support, without you everything would certainly be more complicated. Especially to my mother that was always there for me reminding that I am capable and that has taught me the meaning of hard-work and personal responsibility.

Last but not least, I thank to all my friends who contribute directly to my happiness, I thank you for the support and motivation.

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2017

This thesis was fully performed at the National Reference Laboratory for Gastrointestinal Infections, Department of Infectious Diseases, of the National Health Institute Doutor Ricardo Jorge, Lisbon, Portugal under the direct supervision of Dr. Mónica Oleastro in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon.

Abstract

Clostridium difficile affects patients in hospitals and communities worldwide, is responsible for significant annual mortalities and represents a considerable economic burden on healthcare systems. This bacterium might also be carried asymptomatically in the gut, potentially leading to 'silent' onward transmission. Treatment has always been difficult, because the disease is both caused and resolved by antibiotic intake. The two main *C. difficile* virulence factors are toxins A and B, both of which are pro-inflammatory and enterotoxic in human intestine. Clinically relevant toxin A-negative/toxin B-positive strains that cause diarrhoea and colitis in humans have been isolated with increasing frequency worldwide, namely the multidrug resistant PCR-ribotype (RT) 017. Previous studies documented changes in *C. difficile* infection (CDI) epidemiology associated with the rapid emergence of antibiotic-resistant strains, highlighting the importance of antimicrobial susceptibility surveillance.

The present work describes epidemiological and antimicrobial susceptibility data of *C. difficile* strains isolated in Portugal. A total of 378 *C. difficile* strains from 11 Portuguese hospital centres were characterized regarding toxin profile and RT, and part of these strains were also evaluated for its susceptibility to moxifloxacin, vancomycin, metronidazole, rifampicin and imipenem and determinants of antimicrobial resistance. Multilocus variable tandem repeat analysis (MLVA) and whole genome sequencing (WGS) analysis was also performed in a subgroup of epidemic, multidrug isolates.

Most of the isolates were toxigenic (91.3%), of which 94.2% had both toxins A and B and 25.8% of them also had a binary toxin. Seventy-five different RTs were identified. RT027, RT014, RT106 and RT017 were the most frequently isolated. There was no evidence of resistance to vancomycin among the 183 tested strains, and reduced susceptibility to metronidazole was rare (2.2%). Resistance to moxifloxacin was evident in multiple RTs, and were mainly from RTs positive for the three toxins, RT027 (18/18), RT126 (8/9) and RT078 (6/12), except the RT017 (19/19), which is toxin A-negative/toxin B-positive. All moxifloxacin-resistant strains exhibited a known mutation in GyrA (Thr82Ile). Rifampicin resistance was found in 11.5% of the 183 strains tested, most from RT017 (19/19) but also in one strain from RT241 and other from RT043. Most rifampicin-resistant strains harbour the previously described mutations in RpoB (His502Asn and Arg505Lys), although one mutation, Ser507Leu, found alone in a resistant strain was not previously described. Of the 181 strains belonging to 57 RTs tested for imipenem susceptibility, only strains from RT017 showing high level of resistance to this antibiotic (MIC > 32 mg/L). The resistance determinants, *ermB* and *tetM* genes were present in 34 (10.1%) and in 63 (20.12%) strains, respectively, being that 22 (6.5%) contained both genes.

Twenty strains were toxin A-negative/toxin B-positive, 19 of them belonging to the well-known emerging RT017, 11 from hospital A isolated in a short period of time suggesting that an outbreak have occurred, the remaining eight from hospital B, isolated between 2016 and 2017, where this RT seems to be endemic. Overall, these strains were multiresistant, presenting resistance to six of the 10 antibiotics tested: moxifloxacin, rifampicin, imipenem, tetracycline, clindamycin and erythromycin (these two belonging to the MLS_B group), with high level of resistance. PCR screening of the resistance determinants showed that all strains harboured the *tetM* gene, but only the eight strains from hospital B were positive for *ermB*. Analysis by WGS revealed the presence of the *ermG* gene in the *ermB*-negative/MLS_B-resistant strains. This gene was found to be in a putative mobile element of 63 kb exclusive of the hospital A clonal cluster. Transformation of a susceptible strain (*C. difficile* 630Δ*erm*), with a plasmid containing the *ermG* gene, proved that the presence of this gene provides high resistance to clindamycin and erythromycin in *C. difficile*. Mutations in penicillin-binding proteins were also observed in all imipenem resistant strains. Phylogenetic analysis of single nucleotide polymorphisms

(SNPs) of RT017 isolates collected from 2012 to 2017 revealed three clusters, each from a single hospital. Subtyping by MLVA was also applied to detect the clonal spread of *C. difficile* belonging to toxin A-negative/toxin B-positive, and the results were overall similar with the WGS analysis. In addition, 74 SNPs variations were found among RT017 strains, namely in proteins involved in antimicrobial resistance and in hypothetical proteins.

The current work gives a contribution to the knowledge of the molecular epidemiology and resistance patterns of *C. difficile* in Portugal. The results presented herein alert to the presence of multidrug resistant strains of RT017 in Portuguese hospitals in endemic and outbreak situations, and indicates the need for adequate use of antimicrobial agents, especially carbapenems, whose resistance was only observed among the strains of this emerging RT. The lineage of strains from RT017 appears to be constantly evolving, acquiring new resistance determinants, which highlights the need for continued epidemiological and antimicrobial surveillance.

The wide variety of RTs found suggests that there are other routes of transmission beyond nosocomial transmission, raising concern about the epidemiological change in this pathogen. As such, other potential sources, particularly in animals, which may also act as a reservoir for *C. difficile* and antimicrobial resistance determinants, should be investigated in the future.

Finally, this study provides the basis for investigating important factors for the spread and persistence of toxin A-negative/toxin B-positive strains, such as studies on the importance of proteins that distinguish these strains, namely the hypothetical proteins.

Keywords: *Clostridium difficile* infection, molecular epidemiology, antimicrobial susceptibility, resistance determinants, ribotype 017.

Resumo

Clostridium difficile é uma bactéria Gram-positiva, anaeróbia estrita e formadora de esporos, que coloniza o cólon. A infeção por *C. difficile* (ICD) encontra-se principalmente associada ao meio hospitalar e consumo recente de antibiótico, representando um fardo económico considerável para os sistemas de saúde. No entanto, este quadro tem estado a alterar-se, verificando-se um acréscimo na incidência de infeção em populações anteriormente pensadas em baixo risco e sem contacto prévio com o ambiente hospitalar. Esta bactéria foi descoberta em 1935 como parte da microdiota intestinal normal de recém-nascidos, no entanto, a sua importância em doenças em humanos só foi identificada mais tarde, na sequência de múltiplos trabalhos conduzidos na década de 1970, quando esta patologia se tornou mais frequente devido ao aumento no consumo de antibióticos.

O espectro da doença clínica varia de diarreia leve a megacólon tóxico, perfuração do colón e morte. No entanto, esta bactéria também pode ser transportada de forma assintomática no intestino, potencialmente levando a transmissão silenciosa. O tratamento da ICD sempre foi difícil, porque a doença é causada e resolvida pela toma de antibióticos. Até a recente introdução da fidaxomicina, o tratamento estava limitado a toma de metronidazol e vancomicina.

Nos últimos anos, a ICD surgiu como uma doença proeminente devido a um aumento súbito na ocorrência de surtos, acompanhada por um aumento da gravidade da doença e mortalidade. Esta alteração foi principalmente associada à disseminação de uma estirpe epidémica denominada ribotipo (RT) 027, principalmente caracterizada por uma elevada resistência às fluoroquinolonas, cujo pico de consumo coincidiu com o início da sua propagação epidémica. Ao mesmo tempo, porém, outras estirpes também começaram a emergir com maior virulência.

Os dois principais fatores de virulência, e responsáveis pelo desenvolvimento da doença, são as toxinas A e B, ambas pró-inflamatórias e enterotóxicas no intestino humano, algumas estirpes são também caracterizadas pela produção de uma toxina binária. No entanto estirpes de *C. difficil*e clinicamente relevantes com fenótipo toxina A-negativa/toxina B-positiva que causam diarreia e colite, têm sido isoladas com maior frequência em todo o mundo, nomeadamente pertencentes ao RT017, resistentes a múltiplos antibióticos.

Embora os agentes antimicrobianos sejam fatores de grande relevância para o desenvolvimento da ICD, a resistência da bactéria não é um pré-requisito para tal, podem sim antecipar uma rápida disseminação dessas estirpes resistentes em ambiente hospitalar, uma vez que um fenótipo de resistência pode conferir uma vantagem seletiva significativa dentro do ecossistema intestinal, facilitando o *on-set* da ICD logo no início da toma de antibióticos. Deste modo, em termos de prevenção da ICD, é imperativo limitar a propagação de estirpes resistentes de *C. difficile*, para tal, uma vigilância de fenótipos e genótipos de resistência é essencial.

O presente trabalho tem como objetivo fornecer informação atualizada relativamente à epidemiologia molecular e suscetibilidade antimicrobiana de estirpes de *C. difficile* isoladas de hospitais Portugueses. Para esse fim, um total de 378 amostras de fezes (correspondentes a 374 pacientes diagnosticados com ICD) provenientes de 11 centros hospitalares portugueses foram sujeitas a cultura anaeróbica. As estirpes de *C. difficile* isoladas destas amostras foram caracterizadas em relação ao perfil de toxinas e RTs; um subgrupo dessas estirpes foi também avaliado quanto à suscetibilidade à moxifloxacina, vancomicina, metronidazol, rifampicina e imipenemo. Alguns determinantes de resistência foram também estudados.

A maioria dos isolados eram toxigénicos (91,3%), dos quais 94,2% apresentavam as toxinas A e B sendo que 25,8% desses também possuíam a toxina binária; no total 33 estirpes eram

não-toxigénicas. Foram identificados 75 RTs diferentes, sendo os RTs mais frequentes o RT027 (13,8%), RT014 (8,2%), RT106 (6,1%) e RT017 (5,1%). Não houve evidência de resistência à vancomicina entre 183 estirpes toxigénicas testadas, a menor suscetibilidade ao metronidazol também foi rara verificando-se em apenas 4 estirpes (2,2%). A resistência à moxifloxacina foi evidente em múltiplos RTs, 55 de 183 (30,1%) estirpes testadas apresentaram resistência, pertencentes principalmente a RTs positivos para três toxinas, RT027 (18/18), RT126 (8/9) e RT078 (6/12), exceto o RT017 (19/19), toxina A-negativa/toxina B-positiva. Todas as estirpes resistentes à moxifloxacina continham uma mutação já descrita na GyrA (Thr82Ile). A resistência à rifampicina foi encontrada em 11,5% das 183 estirpes testadas, na sua maioria do RT017 (19/19), mas também numa estirpe do RT241 e uma do RT043. A maioria das estirpes resistentes à rifampicina continham mutações na RpoB já descritas (His502Asn e Arg505Lys); a mutação, Ser507Leu, descrita neste trabalho pela primeira vez, foi a única encontrada numa estirpe resistente do RT043. De 181 estirpes, pertencentes a 57 RTs diferentes, testadas quanto à suscetibilidade ao imipenemo, apenas as estirpes do RT017 (19/19) apresentaram resistência a este antibiótico com alto nível de resistência (concentração mínima inibitória $\geq$ 32 mg/L). Os genes *ermB* e *tetM*, determinantes da resistência aos MLS_B e à tetraciclina, respetivamente, estavam presentes em 34 (10,1%) e 68 (20,12%) das estirpes testadas, respetivamente, sendo que 22 estirpes (6,5%) continham ambos os genes; a maioria das estirpes (258/338) não continham nenhum destes genes nomeadamente, as estirpes do RT027 (40/41). O gene *catD*, que confere resistência ao cloranfenicol, não foi encontrado em nenhuma estirpe.

Foram identificadas 20 estirpes toxina A-negativa/toxina B-positiva, 19 delas pertencentes ao RT emergente RT017, 11 do hospital A isoladas num curto período de tempo sugerindo que ocorreu um surto, as oito restantes do hospital B, isoladas entre 2016 e 2017 onde esta estirpe parece ser endémica. No geral, essas estirpes revelaram-se multi-resistentes, apresentando resistência a seis dos 10 antibióticos testados: moxifloxacina, rifampicina, imipenemo, tetraciclina, clindamicina e eritromicina (os dois últimos pertencentes ao grupo MLS_B). A triagem por PCR dos determinantes de resistência mostrou que todas possuíam o gene *tetM*, mas apenas os oito isolados do hospital B eram positivos para o *ermB*. A análise dos dados gerados por sequenciação total do genoma (WGS) das estirpes *ermB*-negativas/MLS_B-resistentes revelou a presença do gene *ermG*, pela primeira vez descrito em estirpes clínicas toxigénicas de *C. difficile*. Este gene foi encontrado num putativo elemento móvel de 63 kb e somente nas estirpes associadas ao surto no hospital A. Foi possível verificar que este gene está associado a um alto nível de resistência à clindamicina e eritromicina em *C. difficile* através da inserção de um plasmídeo contendo o *ermG* em uma estirpe sensível (*C. difficile* 630Δ*erm*). Outros genes associados a resistências foram também encontrados neste elemento móvel, como os genes *mefA* e *msrD* que conferem resistência aos macrólidos, e um gene que codifica para uma estreptogramina A acetiltransferase que confere resistência à estreptogramina A em outras bactérias, no entanto este grupo de antibióticos não foi aqui testado. Também foram observadas mutações em genes que codificam para as proteínas de ligação à penicilina (PBPs) nas estirpes resistentes ao imipenemo; estas mutações localizam-se próximo dos motivos conservados da PBP1 (Ala555Thr) e da PBP3 (Tyr721Ser).

A análise filogenética de polimorfismos de nucleotídeos únicos (SNPs) de isolados do RT017 obtidos de 2012 a 2017 revelou a existência de três grupos clonais, cada um com isolados de um único hospital. A análise de *multilocus variable number tandem repeat* (MLVA) também foi aplicada para detetar a disseminação clonal de *C. difficile* toxina A-negativa/toxina B-positiva, e os resultados foram concordantes com a análise de WGS. Além disso, 74 variações de SNPs foram encontradas entre as estirpes do RT017, compreendendo mutações sinónimas e não-sinónimas, nomeadamente em proteínas envolvidas em resistência a antimicrobianos e em proteínas hipotéticas.

Em conclusão, embora grande parte do foco de *C. difficile* tenha sido o RT027, novas estirpes virulentas continuam a emergir, o que requer consideração na vigilância futura. Os resultados aqui

apresentados alertam para a presença de estirpes multirresistentes do RT017 nos hospitais portugueses, sugerindo um potencial epidémico para as mesmas. A presença destas estirpes multirresistentes indica a necessidade de uma utilização adequada dos agentes antimicrobianos, nomeadamente dos carbapenemos, cuja resistência só foi observada entre as estirpes deste RT. A linhagem das estirpes do RT017 parece estar em constante evolução, adquirindo novos determinantes de resistência, o que realça a necessidade de uma vigilância epidemiológica e antimicrobiana contínua, bem como a imposição de medidas de prevenção da transmissão de *C. difficile* no contexto hospitalar, nomeadamente através do diagnóstico e tratamento de todos os pacientes com ICD e restrição de alguns antibióticos.

Contudo, a grande variedade de RTs encontrada no geral, sugere que existem outras vias de transmissão para além da transmissão nosocomial, suscitando preocupação com a mudança na epidemiologia deste agente patogénico. Como tal, outras potenciais fontes de infeção, nomeadamente em animais, que também podem atuar como um reservatório de *C. difficile* e de determinantes da resistência antimicrobiana, devem ser investigadas futuramente.

Por fim, este estudo proporciona a base para investigar fatores importantes para a disseminação e persistência de estirpes toxina A-negativa/toxina B-positiva, como por exemplo estudos futuros sobre a importância das proteínas que distinguem estas estirpes, nomeadamente as proteínas hipotéticas.

Palavras-chave: Infeção por *Clostridium difficile*, epidemiologia molecular, suscetibilidade aos antimicrobianos, determinantes de resistência, ribotipo 017.

Publications in the scope of this work

Menezes, J., Santos, A., Isidro, J., Paixão, P., Toscano, C., Oleastro, M. (2017, June). Study of *Clostridium difficile* multiresistant strain from ribotype 017 in a hospital centre in Portugal. Poster session presented at 2nd International Caparica Conference in Antibiotic Resistance, Costa da Caparica, Portugal.

Menezes, J., Isidro, J., Serrano, M., Santos, A., Paixão, P., Toscano, C., Oleastro, M. (2017). Outbreak of a *Clostridium difficile* multidrug resistant strain from ribotype 017 in a hospital centre in Portugal. In preparation.

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List of Symbols and Abbreviations

Δ	deletion	**CO-HCFA**	Community onset health-care facility-associated
%	percentage	**DNA**	Deoxyribonucleic acid
°C	Celsius degree	**EIA**	Enzyme immunoassay
bp	base pair	**EUCAST**	European Committee on Antimicrobial Susceptibility Testing
CFU	colony-forming unit	**GDH**	Glutamate dehydrogenase
h	hour	**HO-HCFA**	Healthcare facility–onset, health-care facility-associated
kb	kilobase	**HP**	Hypothetical protein
L	liter	**HMW**	High-molecular-weight
min	minute	**INSARJ**	Portuguese National Institute of Health Dr. Ricardo Jorge
mg	milligram	**MIC**	Minimum inhibitory concentration
mL	milliliter	**MLS_B**	Macrolide, Lincosamide and Streptogramin B
mm	millimeter	**MLST**	Multilocus sequence typing
ng	nanogram	**MLVA**	Multilocus variable number tandem repeat analysis
no.	number	**NAAT**	Nucleic acid amplification tests
V	volt	**ORF**	Open reading frame
µg	microgram	**PBP**	Penicillin-binding protein
µL	microliter	**PCR**	Polymerase chain reaction
µM	micromolar	**QRDR**	Quinolone resistance--determining region
AMR	Antibiotic resistance	**RNA**	Ribonucleic acid
BHI	Brain Heart Infusion Agar	**RT**	PCR-ribotype
CA	Community-associated	**SNP**	Single nucleotide polymorphism
CCNA	Cell cytotoxicity neutralization assay	**STRD**	Summed tandem-repeat difference
CDI	*Clostridium difficile* infection	**TC**	Toxigenic culture
CDT	*Clostridium difficile* transferase	**VNTR**	Variable-number tandem-repeat
BA	Brucella Agar	**WGS**	Whole-genome sequencing

1. Introduction

1.1. *Clostridium difficile*, the organisms and infection cycle

Clostridium difficile is a Gram-positive bacterium, strict anaerobe, frequently implicated in cases of nosocomial diarrhoea. The infection is caused by toxigenic strains, although there are naturally non-toxin producing strains capable of colonizing their hosts (Martin, Monaghan, & Wilcox, 2016).

C. difficile was recently renamed as *Clostridioides difficile* (Lawson, Citron, Tyrrell, & Finegold, 2016), however the still more familiar designation of *C. difficile* will be used throughout the present document.

This bacterium was initially identified in 1935 as part of the normal gut microbiota of neonates (Hall & O'Toole, 1935). The spectrum of *C. difficile*-associated disease varies from mild diarrhoea to severe colitis, and it may lead to toxic megacolon, perforation of the colon, sepsis and death. Its association with disease was not described until the 1970s following several studies. In those studies, a causal effect of antibiotic exposure and gut diseases was also demonstrated as patients receiving antibiotic treatment developed pseudomembranous colitis (Bartlett, Chang, Gurwith, Gorbach, & Onderdonk, 1978; Tedesco, Barton, & Alpers, 1974).

C. difficile is a spore-forming bacterium, which, in turn, is able to tolerate extreme conditions that the vegetative form cannot, assuming an essential role in the transmission of the pathogen, as well as in environmental persistence. In this way, the spores are considered as the infectious form, since they allow the survival of the microorganism in the acidic environment of the stomach of the host. Upon reaching the duodenum, the spores get into contact with bile acids which along with glycine and some other cofactors initiate germination (Howerton, Ramirez, & Abel-Santos, 2011; Janoir et al., 2013).

The organism is transmitted from person to person by the fecal-oral route. The cycle of *C. difficile* infection (CDI) begins with an uncolonized individual who is exposed to bacterial spores. Then, upon a disturbance of the normal gut microbiota, occurs the colonization and proliferation of *C. difficile*. The infected individual will also be spore excretor, restarting the infectious cycle. The resulting vegetative cells then penetrate the mucus layer and adhere to intestinal epithelial cells (Martin et al., 2016).

Several factors can lead to modification of the intestinal microbiota, such as surgical incisions, nasogastric tube feeding and medication intake. Antibiotic therapy is the major risk factor for CDI because it disrupts the microbial community in the gut that forms a protective barrier, thus weakening resistance to colonization (Dharmarajan, Sipalay, Shyamsundar, Norkus, & Pitchumoni, 2000; Manges et al., 2010).

Pultz and Donskey (2005) have demonstrated that antibiotics can influence the growth of *C. difficile* in different ways depending on its activity against the pathogen and the extent to which the agent disrupts the gut microbiota; CDI can be virtually triggered by any antibiotic, as it promotes damaged in the protective microbiota. However, if the strain of *C. difficile* is resistant to the antibiotic taken it has an additional selective advantage. This is because, even in susceptible strains, the formation of spores allows the bacteria to persist in the gut, germination only occurs when the drug is no longer present at levels sufficient to inhibit growth and multiplication of *C. difficile*. But resistant strains can germinate while the therapy is in progress, so they have an advantage in their spread due to the use of antibiotics (Coia, 2009). In fact, mounting evidence suggests that antibiotic resistance is a key player in the epidemiology of CDI (Gerding, 2004). Fluoroquinolone prescribing correlated highly with incidence of CDI cause by fluoroquinolone-resistant strain, having been observed decline in incidence of CDI

after restriction of this antibiotic (Dingle et al., 2017). Therefore, it is essential to monitor the resistance profile of this microorganism.

1.2. Virulence factors

The main virulence factors associated with *C. difficile* infection are two large enteric toxins, A and B toxins, whose action on the colonic intestinal epithelium are responsible for an intense inflammatory response causing acute inflammation of the large intestine. Those toxins are encoded by two genes, *tcdA* and *tcdB* situated on the *C. difficile* chromosome in a 19.6 kilobase (kb) pathogenicity *locus* (PaLoc), that also contains three additional regulatory genes, *tcdR*, *tcdC* and *tcdE* (Kuehne et al., 2010). TcdR is a sigma factor that facilitates the binding of RNA polymerase to the promoters of the *tcdA* and *tcdB* genes, therefore promoting toxin production (Mani & Dupuy, 2001); TcdC is an TcdR-specific anti-sigma factor that appears to negatively regulate toxin A and B expression through disruption of TcdR interaction with RNA polymerase (Matamouros, England, & Dupuy, 2007); finally, *tcdE* encodes a protein that has similarity to phage holin involved in toxin secretion (Govind & Dupuy, 2012).

Both toxins A and B catalyse the glucosylation and, hence, inactivation of Rho protein family of GTPases in intestinal epithelial cells, mediating disorganization of the cell cytoskeleton and cell death. Structurally these toxins have three other domains: i) the cysteine protease domain, involved in the autocatalytic processing of the toxin protein that releases the enzymatic domain in the cytosol after receptor-mediated endocytosis; ii) the translocation domain that mediates entry of the toxin into the target cell cytoplasm; iii) the receptor-binding domain, responsible for binding to and the uptake into the target cell (Just & Gerhard, 2004; Reineke et al., 2007).

Several studies tried to figure out the relative contributions of both toxins in the pathogenesis; toxin A alone was first believed to induce most of the pathology observed after infection, but Lyras et al. (2009) using isogenic *tcdA* and *tcdB* mutants in a hamster model showed that, in fact, toxin B is essential for virulence while toxin A is dispensable. However the studies conduct by Kuehne et al. (2010) using a similar method re-establish the importance of both toxins A and B in CDI, since they reveal to be important mediators of *in vitro* cytotoxicity and *in vivo* virulence. Many factors differentiate both studies which could lead to different interpretations. Although both groups used derived strains of *C. difficile* 630, these strains had been separately passaged numerous times over a long period of time (>15 years) and there may be genetic differences between them, in fact, the strain used by Kuehne et al. (2010) produces significantly more toxin A than the equivalent strain used in the earlier study (Carter, Awad, Kelly, Rood, & Lyras, 2011; Lyras et al., 2009). The end points used to determine the *in vivo* pathogenicity of the strains were also different in both studies. Death was an endpoint for Lyras et al. (2009) study, by contrast a clinical scoring system comprising weight loss, behavioural changes, and wet tail, followed by sacrifice of moribund and sick animals, was used by Kuehne et al. (2010). In any case, both toxins are essential for disease because non-toxigenic strains are avirulent (Kuehne et al., 2010).

Some strains of *C. difficile* only produce a functional toxin B, due to deletion in the repetitive region of *tcdA* gene (*tcdA*-/*tcdB*+). Studies showed that, toxin B from those strains is able to modify more substrates than wild-type toxin B from toxin A-positive/toxin B-positive (*tcdA*+/*tcdB*+) strains due to polymorphisms in its *tcdB* gene, giving rise to altered glucosylation of Rho proteins and inducing a cytopathic effect on cells (Chaves-Olarte et al., 1999; Rupnik, Kato, Grabnar, & Kato, 2003).

In addition to these two toxins, some strains produce a binary toxin known as *C. difficile* transferase (CDT), encoded by two genes, *cdtA* and *cdtB*, and located in a different *locus*, the Binary

Toxin Encoding *Locus*. CDT induces redistribution of microtubules and formation of long microtubule-based protrusions at the surface of intestinal epithelial cells that increase bacterial adherence; this toxin also causes death of beneficial eosinophils that would play an important role in promoting a healthy immune response during infection (Cowardin et al., 2016; Schwan et al., 2009).

Although the toxins are regarded as the primary virulence factors, other factors intervene in the pathogenic process, notably to allow the establishment of the bacterium in its colonic niche, such as the sporulation. Since *C. difficile* is a strict anaerobe, its virulence is linked to the ability to form spores, being these able to survive in aerobic environments and resist to cleaning measures and disinfection agents (Dawson, Valiente, Donahue, Birchenough, & Wren, 2011; Janoir et al., 2013).

1.3. Epidemiology changing and risk factors

CDI is primarily regarded as a healthcare-related disease that is most prominent among the elderly population, representing a huge clinical and economic burden. This is most likely due to a reduced immune status, concomitant comorbidities and several hospitalizations as these increases the risk of exposure to the microorganism and clinical outcome (Dharmarajan et al., 2000). Also, this population is more prompt to take antibiotics, which are the main risk for CDI. Use of drugs that reduce gastric acid (such as proton pump inhibitors) also increase the likelihood of infection (Janarthanan, Ditah, Adler, & Ehrinpreis, 2012). However, in the past years, the proportion of CDI occurring in patients outside the hospital setting has increased, targeting also healthy young people and peripartum women, two groups previously thought to be at low risk (Centers for Disease Control and Prevention, 2005; van Dorp et al., 2017). *C. difficile* is very widespread in the environment, and can be found in diverse reservoirs such as soils, river waters, lakes, and also in various animal, such as animals in the food chain (cattle, pigs) and also in pets (Álvarez-Pérez, Blanco, Harmanus, Kuijper, & García, 2017; Thitaram et al., 2016); a genomic similarity has been found between strains isolated from humans and from animals, mostly pigs (Janezic, Ocepek, Zidaric, & Rupnik, 2012; Knight, Squire, Collins, & Riley, 2017).

Changing *C. difficile* epidemiology is noted worldwide and is variable across countries, particularly in relation to prevalent strains and its antimicrobial resistance pattern. Historically low rates of severe disease and death (3% or less) may have led to an under estimation of the importance of *C. difficile*-associated disease as a healthcare-associated infection (McDonald et al., 2005); nowadays, on average, seven CDI cases occur for every 10,000-overnight patient stays in European hospitals, making it the most commonly reported pathogen associated with hospital-associated gastrointestinal disease in Europe (Davies et al., 2014). In the United States a population-based study by Lessa et al. (2015) estimated almost a half million cases in 2011, with 83,000 recurrences and 29,300 deaths, while the National and State Healthcare Associated Infections progress report, based on 2014 data, that CDI incidence is higher than methicillin resistant *Staphylococcus aureus* (MRSA) incidence in U.S., and although an 8% decrease in CDI has been observed between 2011 and 2014, U.S. hospitals reported a significant increase in CDI between 2013 and 2014 (Centers for Disease Control and Prevention, 2016). This shift in epidemiology has been noted from 2,000 onwards, when there was a further increase on number and severity of CDI, with higher transmission rates and greater mortality, mainly due to the spread of PCR-ribotype (RT) 027. This epidemic strain is characterized by high resistance to fluoroquinolones, whose peak of consumption in North America coincides with the beginning of its epidemic spread (McDonald et al., 2005; Muto et al., 2005; Pépin, Valiquette, & Cossette, 2005).

In Portugal the first CDI outbreak was reported in 2014 with a mortality rate of 11.3%, been attributed to the RT027 that was also reported as the most frequent RT in the country, mainly due to

healthcare facility-associated cases (Oleastro et al., 2014; Santos et al., 2016).

Although the increased rates of CDI have been primarily attributed to RT027, CDI cases attributed to other emerging RTs, such as RT017 have been reported worldwide (Dobreva et al., 2013; Drudy, Harnedy, Fanning, Hannan, & Kyne, 2007; Lee, Lee, Lee, Riley, & Kim, 2014).

1.4. Toxin A-negative/toxin B-positive ribotypes

A number of *tcdA*-/*tcdB*+ RTs have been identified to date (Rupnik et al., 2003), but the most clinically significant is RT017 (also known as toxinotype VIII) that is been isolated with increasing frequency worldwide.

The first RT017 outbreak reported occurred in a Canadian hospital in 1998 over a three month period (Alfa et al., 2000). Since that time, cases associated with this RT have been described by other groups. In Poland between 2004 and 2006 and in Bulgaria between 2008 and 2012, RT017 was identified to be the most prevalent, infecting 44 and 28% of the patients, respectively (Dobreva et al., 2013; Pituch et al., 2011).

This trend is also observed in Asia, where RT017 is one of the most prevalent RTs. A Korean study, where sampling was performed over a 10-year period, showed that RT017 was the most dominant strain type among hospitalized patients during 2004–2008. These results coincided with the initial use of imipenem and moxifloxacin at the hospitals, making it feasible that the introduction of these drugs would be related to the emergence of this RT, once the resistance levels among their RT017 isolates were 12 and 85% for these drugs, respectively. Additionally, they were also resistant to clindamycin, erythromycin and ciprofloxacin (Lee et al., 2014).

In other studies, the increased prevalence of RT017 strains has also been associated with increased antibiotic resistance. Among the RT017 Polish isolates, 86.8% were resistant to imipenem and 91% were erythromycin-resistant whereas only 7% of the other RTs with the most common toxin profile (*tcdA*+/*tcdB*+) exhibited resistance to this antibiotic (Pituch et al., 2011). Isolates from an outbreak in Ireland were all resistant to multiple antibiotics including macrolides, lincosamide and newer classes of fluoroquinolones (Drudy et al., 2007). In Portugal RT017 is one of the most common RTs, having been isolated endemic multidrug resistant strains in one hospital (Isidro et al., 2017; Santos et al., 2016).

Although RT017 strains are negative for the toxin A encoding gene *tcdA*, they are capable of causing the full spectrum of clinical illness usually associated with *tcdA*+/*tcdB*+ strains (ranging from asymptomatic colonization through to severe disease), and cases of fulminant colitis have been documented (Alfa et al., 2000; Arvand, Hauri, Zaiss, Witte, & Bettge-Weller, 2009; Drudy et al., 2007).

The *C. difficile* strain M68 is the representative strain of the RT017, and has the full genome sequenced. The *tcdA* gene of this type strain contains a 1.8 kb deletion at the 3′ end and a nonsense mutation at the amino acid 47 (He et al., 2010; Rupnik et al., 2003).

1.5. Diagnosis and treatment

There are many differences in CDI surveillance systems, infection detection and laboratory diagnosis between and within countries (Davies et al., 2014). Two reference tests are available, the cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC). TC demonstrates the presence of *C. difficile* isolates with the ability to produce toxin when cultured, while CCNA detects the presence of toxin in stool samples. Both these techniques are however time consuming, requiring specialized staff and equipment. Therefore, in hospital laboratories other standards tests are

often used for patients with diarrhoea, such as the toxin A/B commercial enzyme immunoassay (EIA) that directly detects *C. difficile* toxins in stool samples or EIAs that detect the glutamate dehydrogenase (GDH), an enzyme that is produced by both toxigenic and non-toxigenic strains. Nucleic acid amplification tests (NAAT), such as PCR assay and loop-mediated isothermal amplification of DNA, targeting toxin genes, are also used. However, all these tests have poor diagnostic accuracy as single tests and are no longer recommended as a stand-alone test to diagnose CDI (Crobach et al., 2016; Planche et al., 2013).

One strategy to overcome this problem is to use two- or three-step screening algorithms. In this approach, an initial test is performed, which can be GDH EIA or nucleic acid amplification tests (NAAT), and if negative, specimens are reported as negative with no further testing done. Positive specimens must undergo additional testing for *C. difficile* by toxin A/B EIA and eventually for TC or NAAT (in case first test was a GDH EIA test) when toxin A/B EIA result is negative (Crobach et al., 2016).

Because many cases of CDI are hospital acquired, once CDI is diagnosed in a patient immediate implementation of appropriate infection control measures such as appropriate handwashing, gloving and improved environmental decontamination is mandatory to prevent further spread within the hospital (Vonberg et al., 2008).

The mainstay of treatment is discontinuation of the offending antibiotic and administration of metronidazole or vancomycin; oral metronidazole is indicated in cases of mild to moderate disease, oral vancomycin for serious CDI, and combination therapy with enteral (oral/intracolonic) vancomycin and intravenous metronidazole in cases of ileus or toxic megacolon. When oral treatment is not possible, parenteral metronidazole is recommended, preferably combined with intracolonic or nasogastric administration of vancomycin. For severe, complicated and recurrent CDI cases, treatment with fidaxomicin is recommended, and has been found to be at least as effective as vancomycin and may be more effective for achieving symptomatic cure, since also targets the spores of *C. difficile*. Faecal transplantation following antibiotic treatment with an oral glycopeptide is reported to be highly effective in treating multiple recurrent CDI. For relapsing CDI, rifaximin use is also proposed as a chaser therapy (Debast et al., 2014; Nelson, Suda, & Evans, 2017).

1.6. Antimicrobial susceptibility and mechanisms of resistance

Apart from the classic virulence determinants, other factors, such as antibiotic resistance, promotes increased pathogenicity and spread of *C. difficile* strains. This pathogen presents an enormous capacity of adaptation to the environment, being able to become resistant to multiple drugs through metabolic and genetic alterations, which includes mutations in specific genes and gene acquisition. In fact, in a study conducted in 14 European countries, 47% of the strains analysed were resistant to at least one antibiotic and multidrug resistant was observed among the predominantly RTs as result of antibiotic-selective pressure (Spigaglia et al., 2011).

Antibiotic resistance may be generated by different mechanisms and the surveillance of those different mechanisms and respective susceptibility patterns, for drugs frequently used in hospital environment and in the community, are needed.

The resistance mechanism for vancomycin, the first-line of treatment for severe CDI, is still unclear, with only a few strains reported with reduced susceptibility to this drug (Freeman et al., 2015). In *in vitro* studies, *C. difficile* mutants exhibiting decreased susceptibility to vancomycin harboured mutations leading to a P108L substitution in *murG* gene, responsible for the conversion of lipid I to lipid II during the stage of peptidoglycan biosynthesis where the antibiotic acts, which could lead to antibiotic

resistance. Nonsense mutation in an RNA/single-stranded DNA exonuclease (homologous *locus* tag CD630_3659 in *C. difficile* 630), missense mutation causing a Asp244Tyr substitution in the β-subunit of RNA polymerase (encoded by *rpoC*) and a single amino acid deletion in L-serine dehydrogenase (encoded by *sdaB)* were also observed in those mutants (Leeds, Sachdeva, Mullin, Whitney Barnes, & Ruzin, 2014).

C. difficile strains showing resistance to metronidazole are rare and when observed, the phenotype has been transient and lost after exposure of the bacteria to freeze. Therefore, the investigation of resistance mechanisms has proved to be difficult. Studies suggest that different *C. difficile* strains resistant to metronidazole can show peculiar alterations in their enzymes or metabolic pathways, such as decrease in the concentration of aminoacyl-tRNAs proteins, which likely result in post translational variations of proteins relevant for metronidazole activation, increased expression of proteins involved in DNA repair and variations in the electron transport, that could lead to alterations in both the energy production and intracellular redox potential, which influences the efficiency of metronidazole entry in the bacterial cell (Lynch et al., 2013; Moura et al., 2014).

Genetic mutations are involved in resistance to both fluoroquinolones and rifamycin. Resistance to fluoroquinolones in *C. difficile* is due to point mutations in the quinolone-resistance determinant region (QRDR) of GyrA or GyrB, the DNA gyrase subunits target of these agents. Several amino acid substitutions have been identified in both GyrA and/or GyrB among moxifloxacin-resistant strains (Spigaglia et al., 2008). Rifamycin's are a class of antibiotics including rifampicin that inhibit the RNA synthesis. Resistance to this group of antibiotics are associated with point mutations in *rpoB*, the gene encoding for the β-subunit of RNA polymerase, thus changing the antibiotic target and preventing its action (Curry et al., 2009). Resistance rates varied by geographic locations (Freeman et al., 2015), however rifamycin agents have been proposed as therapy for treatment of relapsing CDI (Debast et al., 2014).

Recently, mutations in two high-molecular-weight (HMW) penicillin-binding proteins (PBPs) were suggest as being involved in imipenem resistance in *C. difficile* strains (Isidro et al., 2017). This carbapenem antibiotic binds to PBPs inhibiting peptidoglycan synthesis, so these mutations would affect the antibiotic binding to its targets. Reports of imipenem resistance have been attributed to RT017 strains which have an additional PBP that could also play an important role in carbapenem resistance (Isidro et al., 2017; Lee et al., 2014).

Resistance to macrolide-lincosamide-streptogramin B (MLS_B) group of antibiotics is a common phenotype in certain RTs, most notable among RT017, RT001, RT012, RT046, RT126 , RT053, and RT078 (Freeman et al., 2015; Tenover, Tickler, & Persing, 2012). In *C. difficile*, MLS_B is highly related to the presence of the *ermB* gene carried by mobile elements (Spigaglia & Mastrantonio, 2004). The *ermB* gene encodes for an rRNA methyltransferase that catalyse the methylation of specific adenine residue in 23S rRNA, altering the antibiotic binding site (Leclercq, 2002). However, *ermB* was not identified in all *C. difficile* clinical strains expressing high-level resistance to either erythromycin or clindamycin, two antibiotics belonging to MLS_B group (Nyc et al., 2016; Spigaglia et al., 2011).

Recently, a *cfr*-like gene named *cfrC* was described in *C. difficile* strains, and appears to be associated with a transposon similar to Tn6218, a novel Tn916-like transposon (Candela, Marvaud, Nguyen, & Lambert, 2017; Marín et al., 2015). This gene encodes for a 23S rRNA methyltransferase which causes C-8 modification in A2503 located in the peptidyl transferase region of bacterial ribosome, which modifies the antibiotic target. This mechanism confers resistance to several antimicrobial classes comprising phenicol's (as chloramphenicol), oxazolidones and pleuromutilins, lincosamides and streptogramin A drugs (Candela et al., 2017).

Besides the *cfr*-like gene, chloramphenicol resistance is usually due to the presence of the *catD* gene, which encodes for a chloramphenicol acetyltransferase (CAT) inactivating the antibiotic, and is usually located in the Tn4451/Tn4453 transposons family (Spigaglia et al., 2011). This phenotype is not very common in *C. difficile* clinical strains, varying between countries (Freeman et al., 2015).

Resistance to tetracycline in *C. difficile* is also related to a transposon-associated resistance determinant, which is, in most of the cases, the *tetM* gene, encoding for a ribosomal protection protein that binds to the ribosome and thereby preventing the drug from attaching to its binding site (Dönhöfer et al., 2012; Spigaglia, Carucci, Barbanti, & Mastrantonio, 2005). In *C. difficile, tetM* is usually found on conjugative Tn916-like elements and Tn5397 (Spigaglia et al., 2005). These two transposons functionally differ from each other by its integration/excision module; Tn5397 harbours the *tndX* gene that appears to encode a member of the large resolvase family of site-specific recombinases. The Tn916 harbours the the *int* and *xis* genes that are required for its excision and integration (Spigaglia et al., 2005; H. Wang et al., 2000). The widespread use of tetracyclines during the past 60 years has led to an increase in acquired tetracycline resistance determinants among clinically important pathogenic bacteria, making important to study the susceptibility pattern of this antibiotic (Dönhöfer et al., 2012). In *C. difficile*, resistance to tetracyclines varies widely between countries and with RT. In a European study published in 2007, tetracycline resistance was observed in *C. difficile* in isolates from the UK and the Netherlands, 14.3% resistance in isolates from Poland, 21.4% resistance in isolates from Hungary, and 38.9% resistance in isolates from Greece; compared to 9.2% across the entire study (Barbut et al., 2007).

2. Aims and objectives

C. difficile is well recognized as the leading cause of antibiotic-associated diarrhoea, having a significant impact in both healthcare and community settings. In view of this situation, knowledge about the circulation dynamics of different strains in Portugal is of great importance. Modern technologies, such as whole-genome sequencing and multilocus variable number tandem repeat analysis, are helping to track *C. difficile* transmission.

In order to contribute to the knowledge about the current paradigm of CDI in the country, it was intended with this work to accomplish the following objectives:

(i) Identify and characterize strains isolated from Portuguese hospitals, through phenotypic and molecular techniques, namely the identification of predominant RTs and detection of main virulence factors;

(ii) Evaluate antimicrobial susceptibility patterns, identify mechanisms of resistance and investigate possible associations of specific genotypes with antimicrobial resistance phenotypes;

(iii) Evaluate the frequency of toxin A-negative/toxin B-positive strains and their epidemiological characteristics;

(iv) Perform a molecular study in order to unravel relevant features of this group of strains as well evaluate clonal relationships;

(v) Correlate genotypic and phenotypic data regarding antimicrobial resistance through a whole genome approach.

3. Methods

3.1. Samples and data collection

Between October 2015 and June 2017, the National Reference Laboratory for Gastrointestinal infections (NRL_GI) from the Portuguese National Institute of Health Dr. Ricardo Jorge (INSARJ) received, under the scope of CDI surveillance, stool samples from patients with suspected CDI from 11 Portuguese hospital centres. The samples had positive enzyme immunoassay (EIA) test for the presence of toxin A/toxin B and/or positive EIA for GDH. Each sample was accompanied by a short questionnaire on the patient's clinical and epidemiological data, including age, sex, place (hospital or community) and time of symptoms onset. CDI cases were classified as healthcare facility–onset healthcare facility-associated (HO-HCFA) when the symptoms onset occurred > 48 h after admission to a health-care facility, community-associated (CA) when the onset of symptoms occurred outside a health-care facility or less than 48 h before admission, and unknown when no data was available (Cohen et al., 2010). A total of 455 samples were studied.

3.2. *Clostridium difficile* culture

Faecal samples were subject to alcohol shock treatment: 1 g of stool was mixed with 1 mL of ethanol 100%; for liquid stools, 1 mL was mixed with 1 mL of ethanol, and left to rest for 60 min at room temperature. Then, 1-2 drops of the faeces/ethanol suspension were inoculated on a selective chromogenic medium chromID™ *C. difficile* agar (CDIF) (bioMérieux) for the growth of *C. difficile.* The CDIF plates were examined after incubation at 37 °C for 48 h, under anaerobic atmosphere generated by Anoxomat™ (MART Microbiology BV) with a catalyst.

Suspected *C. difficile* colonies were selected based on colour and morphology (5-7 mm in diameter, flat with a filamentous edge); for each isolate one colony was cultured onto a Brain Heart Infusion (BHI) agar plate and its identity confirmed by PCR targeting the glutamate dehydrogenase gene (*gluD* gene).

3.3. Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed for the antibiotics moxifloxacin (5 μg), vancomycin (5 μg), metronidazole (5 μg) and rifampicin (5 μg) by disk diffusion (Oxoid). For a subgroup of strains, the minimum inhibitory concentration (MIC) of imipenem, chloramphenicol, tetracycline, tigecycline, clindamycin and erythromycin were determined by diffusion gradient, using Etest® strips (bioMérieux) according to the manufacturer's instructions. Briefly, one colony from each cultured sample was selected and subcultured on BHI plates and incubated anaerobically at 37 °C for 24 h. Overnight cultures of *C. difficile* strains were suspended in Schaedler Broth with Vitamin K1 (BD, BBL™) to a density equivalent to 1.0 McFarland (~$3x10^8$ CFU/mL), and then a sterile cotton swab was used to spread the inoculum evenly onto the Brucella Agar (BA) supplemented with 5% Sheep Blood, Hemin and Vitamin K1 (BD, BBL™) plates. After the surface was completely dry, antibiotic disks or Etest® strips were applied to each plate. The plates were incubated anaerobically at 37 °C for 24 h for the ones containing the disks and the imipenem Etest® strips, and for 48 h for the remaining plates containing Etest® strips. For antibiotic disks, the following zone diameter ecological cut-off proposed by Erikstrup et al. (2012) was used: ≥ 20 mm, ≥ 23 mm and ≥ 19 mm, to moxifloxacin, vancomycin, metronidazole respectively. For rifampicin, and in order to make the transition to disk diffusion, a comparative test was performed using both antibiotic disks (5 μg) and Etest® strips, and strains were categorized as susceptible if inhibition zone diameter was ≥ 30 mm, corresponding to MIC < 0.004 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

guidelines (2017, http://www.eucast.org/clinical_breakpoints/); resistant strains all had inhibition zone diameter ≤ 6 mm, corresponding to MIC > 32 mg/L.

MICs breakpoints were defined according to Freeman et al. (2015) for: imipenem (≥ 16 mg/L), chloramphenicol (≥ 32 mg/L), tigecycline (> 0.25 mg/L) and for clindamycin (≥ 8 mg/L), and according to Clinical and Laboratory Standards Institute (CLSI) breakpoint guideline (M11-A7, 2012) for tetracycline (≥ 16 mg/L) and erythromycin (≥ 8 mg/L). To optimize growth of *C. difficile*, all necessary medium were reduced for 18–24 h in an anaerobic atmosphere before use (CLSI, 2012). The preparation of inoculum, inoculation and incubation was accomplished within 30 min in order to avoid prolonged exposure to aerobic atmosphere.

3.4. Molecular characterization of strains

DNA was extracted from a 24 h culture using the NucliSENS® easyMAG® (bioMérieux) automated system (bioMérieux) according to the manufacturer's instructions. In brief, samples were placed in the sample vessel and were followed by lysis incubation. Magnetic silica was added to the samples followed by automatic extraction with the Generic 2.0.1 protocol.

The primers used in this study, their respective sequences, targets and references are presented in Table 3.1. The primers' final concentrations used in the PCR mixtures, the expected PCR product length, annealing temperature and gel electrophoresis conditions are all described in Table 3.2.

3.4.1. Identification, toxin profiling and ribotyping

The extracted DNA was used for amplification of *tcdA*, *tcdB*, *cdtA*, *cdtB*, toxin-encoding genes as well as of for the *gluD* gene of *C. difficile* in a single multiplex PCR by using the respective primers listed in Table 3.1. In order to investigate deletions in *tcdA* gene repeating regions, an additional PCR was performed, using the primer pair NKV011/NK9, resulting in a 2535 bp amplicon when no deletion is present, and 1700-1800 bp amplicon when deletions are present.

Multiplex PCR was carried out using HotStarTaq Master Mix (Qiagen) while PCR targeting only the *tcdA* gene was carried out using RANGER DNA Polymerase (Bioline).

PCR ribotyping of isolates was determined using the primer set described by Bidet, Frédéric, Valérie and Petit (1999) and a capillary gel electrophoresis-based approach as described by Indra et al. (2008). The reaction was performed using KAPA2G Robust HotStart DNA Polymerase (Kapa Biosystems) and employed a 16S rRNA primer labelled at the 5' end with 6-carboxyfluorescein (6-FAM). The PCR RTs were determined by uploading the data to the online database Webribo (https://webribo.ages.at/).

3.4.2. Detection of genes and point mutations associated with antibiotic resistance

The primer pairs E5-E6, CL1-CL2 and TETMd/TETMr were used to identify the presence of *ermB*, *catD* and *tetM* gene, respectively. For identification of transposons carrying the *tetM*, the *int* and the *tndX* genes were used as markers of Tn916 and Tn5397 elements, respectively, according to Spigaglia et al. (2005).

BioTaq DNA polymerase (Bioline) was used for screening of these genes and transposons carrying the *tetM*.

Point mutations associated with resistance to rifampicin and moxifloxacin were screened on *rpoB* and on *gyrA*/*gyrB* genes, respectively (Curry et al., 2009; Dridi, Tankovic, Burghoffer, Barbut, &

Table 3.1. Primers used in this study and their respective sequences, targets and references.

Primer name	Sequence (5'-3')	Target	Source
tcdA-F3345	GCATGATAAGGCAACTTCAGTGGTA	*tcdA*	Persson, Torpdahl, & Olsen (2008)
tcdA-R3969	AGTTCCTCCTGCTCCATCAAATG		
tcdB-F5670	CCAAARTGGAGTGTTACAAACAGGTG	*tcdB*	
tcdB-R6079A	GCATTTCTCCATTCTCAGCAAAGTA		
tcdB-R6079B	GCATTTCTCCGTTTTCAGCAAAGTA		
cdtA-F739A	GGGAAGCACTATATTAAAGCAGAAGC	*cdtA*	
cdtA-F739B	GGGAAACATTATATTAAAGCAGAAGC		
cdtA-R958	CTGGGTTAGGATTATTTACTGGACCA		
ctdB-F617	TTGACCCAAAGTTGATGTCTGATTG	*cdtB*	
cdtB-R878	CGGATCTCTTGCTTCAGTCTTTATAG		
Cdiff_GluDF1	GTCTTGGATGGTTGATGAGTAC	*gluD*	Paltansing et al. (2007)
Cdiff_GluDR1	TTCCTAATTTAGCAGCAGCTTC		
NKV011	TTTTGATCCTATAGAATCTAACTTAGTAAC	*tcdA*	Kato et al. (1999)
NK9	CCACCAGCTGCAGCCATA		
16S	6 FAM-GTGCGGCTGGATCACCTCCT	16S-23S rRNA	Bidet et al. (1999)
23S	CCCTGCACCCTTAATAACTTGACC		
E5	CTCAAAACTTTTTAACGAGTG	*ermB*	Spigaglia & Mastrantonio (2004)
E6	CCTCCCGTTAAATAATAGATA		
CL1	ATACAGCATGACCGTTAAAG	*catD*	
CL2	ATGTGAAATCCGTCACATAC		
TETMd	TGGAATTGATTTATCAACGG	*tetM*	Marchese et al. (1998)
TETMr	TTCCAACCATACAATCCTTG		
INTf	GACTGGAGAGAGCCAACGAA	*int*	Spigaglia et al. (2005)
INTr	CATCATGCCGTTGTAATCAC		
tndx1	TACATTGTTAAAACAGCAAGC	*tndX*	
tndx3	TATCAATGAGACACTGCTA		
CDrpoB2-F	ATGGAAGCTATAACGCCTCAA	*rpoB*	Curry et al., (2009)
CDrpoB2-R	ACAGCACCATTTACAGTTCTA		
gyrA1	AATGAGTGTTATAGCTGGACG	*gyrA*	Dridi et al. (2002)
gyrA2	TCTTTTAACGACTCATCAAAGTT		
gyrB1	AGTTGATGAACTGGGGTCTT	*gyrB*	
gyrB2	TCAAAATCTTCTCCAATACCA		
A6Cd-F	6 FAM-TTAATTGAGGGAGAATGTTAAA	A6Cd	van den Berg, Schaap, Templeton, Klaassen, & Kuijper (2007)
A6Cd-F	AAATACTTTTCCCACTTTCATAA		
B7Cd-F	6 FAM-CTTAATACTAAACTAACTCTAACCAGTAA	B7Cd	
B7Cd-R	TTATATTTTATGGGCATGTTAAA		
C6Cd-F	HEX-GTTTAGAATCTACAGCATTATTTGA	C6Cd	
C6Cd-R	ATTGGAATTGAATGTAACAAAA		
E7Cd-F	HEX-TGGAGCTATGGAAATTGATAA	E7Cd	
E7Cd-R	CAAATACATCTTGCATTAATTCTT		
G8Cd-F	6 FAM-TGTATGAAGCAAGCTTTTTATT	G8Cd	
G8Cd-R	AATCTAATAATCCAGTAATTTAAATT	G8Cd	Tanner et al. (2010)
CDR60-F	JOE-AGTTTGTAGGGAAGTGTGTAAATAGAT	CDR60	
CDR60-R	CGCATTAAATTTCACTCCTCAT		

Petit, 2002).

Fragments of those genes were amplified with BIO-X-ACT Short DNA Polymerase (Bioline) followed by Sanger sequencing using the forward primers, CDrpoB2-F, gyrA1 and gyrB1, respectively. Before sequencing, all PCR products were treated with ExoSAP-IT (USB Corporation) according to the manufacturer's instructions. Capillary sequencing was performed with a BigDye terminator Reaction in an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Table 3.2. Primer pairs used in this study, final concentration used in PCR mixture, PCR annealing temperature, amplicon size and electrophoresis conditions.

Primer pair	**Primer concentration** (µM)	**PCR annealing temperature**	**Amplicon size** (bp)	**Electrophoresis conditions** (agarose gel %; time; voltage)
tcdA-F3345/ tcdA-R3969	0.67	54 ºC	629	2.5%; 40 min; 100 V
tcdB-F5670 tcdB-R6079A/ tcdB-R6079B	0.44 0.22		410	
cdtA-F739A/ cdtA-F739B cdtA-R958	0.056 0.11		221	
ctdB-F617/ cdtB-R878	0.11		262	
Cdiff_GluDF1/Cdiff_GluDR1	0.67		158	
NKV011/ NK9	0.625	58°C	2535/1700-1800[a]	1.5%; 40 min; 100 V
16S/ 23S	0.25	54°C	-	-
E5/ E6	0.75	50°C	711	1.5%; 30 min; 100 V
CL1/CL2	0.75	50°C	500	1.5%; 30 min; 100 V
TETMd/ TETMr	0.75	50°C	1080	1.5%; 30 min; 100 V
INTf/ INTr	0.75	50°C	925	1.5%; 30 min; 100 V
tndx1/ tndx3	0.75	50°C	1608	1.5%; 30 min; 100 V
CDrpoB2-F/ CDrpoB2-R	0.5	52°C	485	1.5%; 30 min; 100 V
gyrA1/ gyrA2	0.5	50°C	390	1.5%; 30 min; 100 V
gyrB1/ gyrB2	0.5	50°C	390	1.5%; 30 min; 100 V
VNTR primer pairs	0.22	51°C	-	-

[a] Amplicon size if no *tcdA* deletion is present/if deletion is present.

3.4.3. Multilocus variable number tandem repeat analysis

Six variable-number tandem-repeat (VNTR) *loci* amplification (A6Cd, B7Cd, C6Cd, E7Cd, G8Cd and CDR60) were carried out for all RT017 and RT265 isolates using the primer set listed in Table 3.1, developed by van den Berg, Schaap, Templeton, Klaassen and Kuijper (2007) and Tanner, Hardy and Hawkey (2010) for multilocus variable number tandem repeat analysis (MLVA). Briefly, each forward primer was fluorescently labelled at 5' end with either 6-carboxyfluorescein (6-FAM), hexachlorofluorescein (HEX) or 4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) (Table 3.1). PCR amplifications were performed using KAPA2G Robust HotStart DNA Polymerase (Kapa Biosystems). The repeat numbers for each *locus* were determined by capillary electrophoresis using an ABI 3130xl Genetic Analyzer (Applied Biosystems) and the results used to generate a dissimilarity matrix based on the Manhattan distance between isolates. The dissimilarity matrix was used to construct a minimum spanning tree using the summed absolute distance as coefficient in the BioNumerics (version 3.5)

software (Applied Maths, Sint-Martens-Latem, Belgium). The minimum spanning tree was built with VNTR information of RT017 strains from INSARJ database.

For each *locus*, the summed tandem-repeat difference (STRD) were also calculated and MLVA types associated based on the smallest STRDs: isolates with STRD ≤ 10 were considered genetically related regardless the number of different *loci*; the clonal complexes were defined by a STRD ≤ 2 between two isolates that were either single or double *locus* variants of each other.

3.5. Whole Genome Sequencing

Whole genome sequencing (WGS) was performed for eight RT017 strains and one RT265 strain for genomic comparison between the two groups.

For each strain, DNA sample was extracted using the Isolate II Genomic DNA kit (Bioline, UK) according to the manufacturer's protocol, and further quantified using a fluorometric method (Qubit dsDNA HS Assay Kit). For each isolate, paired-end (2 x 250 bp) WGS was carried out in a MiSeq equipment (Illumina Inc., CA, USA) available at INSARJ. FastQC version 0.11.5 was subsequently applied to check the reads' quality before and after quality improvement measures carried out using Trimmomatic version 0.36. Then, high-quality processed reads were subjected to *de novo* genome assembly using SPAdes (version 3.10.1). Draft genome sequences were analysed in order to: i) perform genome annotation to identify functional properties and biological roles of genes using RAST server version 2.0 (http://rast.nmpdr.org/); ii) perform *in silico* Multilocus Sequence Typing (MLST) and allele determination of well-known virulence-associated genes, using the online platform available at PUBMLST (http://pubmlst.org/); iii) search for the presence of putative antimicrobial resistance (AMR) genes using both CARD (https://card.mcmaster.ca/) and ResFinder version 2.1 (http://www.genomicepidemiology.org/) database; iv) identify potential dissimilarities enrolling AMR genes; and v) verify the genomic context of potential horizontally-transferable AMR genes. Open reading frames (ORFs) visualisation was conducted using SnapGene Viewer (version 4.0.5) software.

Sequence reads (after quality improvement using Trimmomatic) of all *C. difficile* RT017 clinical isolates were mapped to the published genome of the RT017 reference strain, *C. difficile* M68 (GenBank accession number: NC_017175) using Snippy v3.1 tool (https://github.com/tseemann/snippy); base-pair calls at each position in the genome were used to identify single nucleotide polymorphisms (SNP) between the clinical isolates and the reference genome. MEGA5 software (http://www.megasoftware.net) was applied to calculate matrices of nucleotide distances. RAxML (NG v0.4, beta) was applied to perform maximum likelihood phylogenetic reconstructions over the obtained core-genome SNP alignment (enrolling 74 variant sites) by using the general time-reversible model (GTR+G) with bootstrapping (1000 replicates). Microreact platform (https://microreact.org/) was used to visualize the phylogenetic three linked to antimicrobial resistance data.

3.6. Cloning and expression of *ermG* gene

In order to verify if the presence of *ermG* gene by itself leads to MLS_B resistance, a multicopy plasmid carrying the gene was inserted in *C. difficile* 630Δ*erm*, and antimicrobial susceptibility of the transformed strains were evaluated.

To express the *ermG* gene under the control of a *Ptet* inducible promoter, the *ermG* gene with its ribosome-binding site (positions -12 to +793 from the translational start codon) was amplified using primers ermG850D (GGATTCGGAGAGGTTATAATGAACAAAG) and ermG1660R (ATAGTTTAGCGGCCGCATTTTAACTTATGCTACCCTACC). The resulting PCR product (810 bp) was digested with EcoRI and NotI and cloned into pAM25, a derivative of pRPF185 lacking the

gusA gene (Fagan & Fairweather, 2011). Using the *Escherichia coli* HB101 (RP4) strain containing either pRPF185 or pMS534 (pAM25-ermG), these plasmids were transferred by conjugation into *C. difficile* 630Δ*erm*.

Transformable strains were incubated at 37°C for 24 h in BHI plates with 15 µg/mL of thiamphenicol. Overnight cultures were suspended in Schaedler Broth with Vitamin K1 (BD, BBL™), to a density of 1.0 McFarland and mixture with 250 ng/mL of anhydrotetracycline and 15 µg/mL of thiamphenicol; 500 µL of the mixture were scattered onto BA plates, and the excess suspension was aspirated with a sterile disposable Pasteur pipette. MIC determination of erythromycin and clindamycin was performed using Etest® strips (bioMérieux). A *C. difficile* 630Δ*erm* strain carrying the multicopy plasmid, but without the *ermG* insertion was used as control.

The Sanger sequencing, capillary electrophoresis and WGS were performed at the *Unidade de Tecnologia e Inovação*, of the *Departamento de Genética Humana*.

The construction of *ermG* inducible strains was carried at the Microbial Development Lab, *Instituto de Tecnologia Química e Biológica - António Xavier, Universidade Nova de Lisboa*, Portugal.

4. Results

4.1. Toxin profile and ribotyping of *Clostridium difficile* clinical strains

Of the 455 samples sent to the Reference Laboratory of INSARJ, a total of 378 *C. difficile* strains were recovered after anaerobic culture and identification by PCR targeting *gluD*.

Overall, 345 isolates (91.3%) were toxigenic and 33 (8.7%) were non-toxigenic strains. Among the 325 strains with the genotype *tcdA*+/ *tcdB*+, 89 harboured also the CDT-encoding genes (*cdtA*+/ *cdtB*+). Specific PCR for toxin A revel that some of the strains characterized as *tcdA*+ by the multiplex had a large deletion in the gene, thereby, 20 isolates were identified as *tcdA*-/*tcdB*+, none of them were positive for *cdtA*/*cdtB* (Table 4.1).

Table 4.1. Toxin gene contents of 345 toxigenic *Clostridium difficile* clinical isolates.

Toxin gene, isolates No. (%)	CDT	Isolate no. (%)
tcdA+/ *tcdB*+, 325 (94.2)	+	89 (25.8)
	-	236 (68.4)
tcdA-/*tcdB*+, 20 (5.8)	-	20 (5.8)

CDT, *C. difficile* transferase.

Concerning RTs, lack of amplification was observed for two isolates; in the remaining 376 isolates, a high diversity was observed (Figure 4.1).

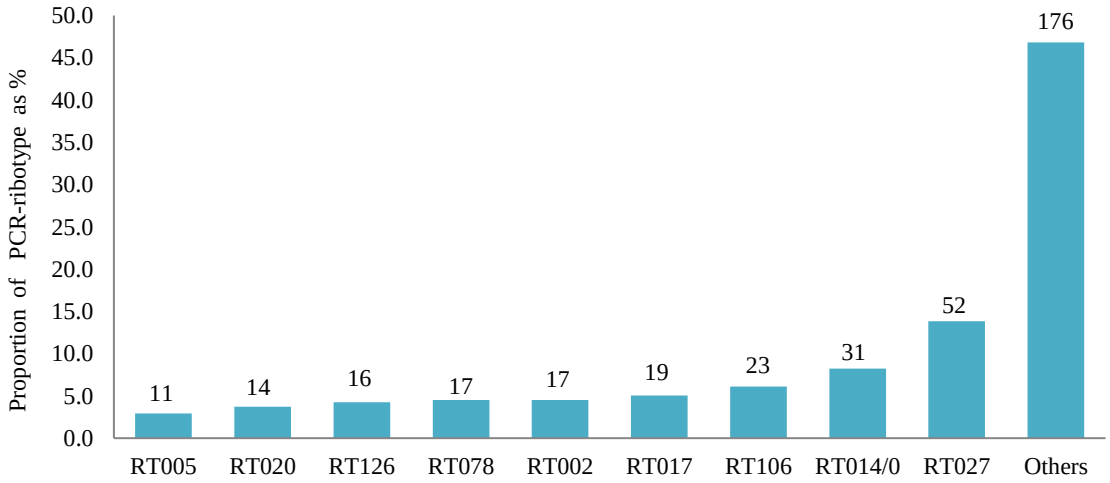

Figure 4.1. Distribution of PCR-ribotypes among 376 *Clostridium difficile* clinical strains isolated from eleven Portuguese hospital centres during 2015-2017. "Others" includes 66 RTs with less than 10 isolates each. The number of strains are represented on top of the bars.

RT027 was the most prevalent (13.8%), followed by RT014 (8.2%) and RT106 (6.1%). The percentage of RT017 was 5.1%, being the fourth most representative RT, followed by RT078 and RT002 (4.5%), RT126 (4.3%), RT020 (3.7%) and RT005 (2.9%). Sixty-six less common RTs accounted for 46.8% of the total were found, adding a total of 75 different RTs among 376 isolates.

Three of the most frequent RTs were positive for *tcdA*, *tcdB* and also *cdtA*/*cdtB* (RT027, RT078 and RT126), while the 19 strains from RT017 were *tcdA*-/*tcdB*+, as well a single strain from RT265.

The distribution of the RTs varied widely between and within hospitals. However, 11 of the 19 isolates from RT017 were detected in 11 patients hospitalized in the same ward of the same hospital (herein designated as Hospital A), with the following temporal distribution: 1 patient in January, 6 patients in February, 1 in March, 1 in April, 1 in June and 1 in July, all in 2016, suggesting the occurrence of nosocomial transmission.

4.2. Patients characteristics and clinical data

The 378 *C. difficile* strains were isolated from 374 different patients, as four patients were colonized by two different strains (two different RTs). Table 4.2 summarize the relevant demographic and clinical data of the patients.

The ratio female:male was 1.3 and the majority of CDI cases (70.1%) was from older patients (age ≥ 65 years).

Most cases corresponded to hospital-acquired CDI (63.1%) but there were 90 cases associated with the community setting (24.1%) and 48 cases (12.8%) classified as unknown due to lack of information.

Table 4.2. Demographic and clinical data of patients with positive culture for *Clostridium difficile.*

Patients characteristics		**no. (%)**
Gender (n = 371)	Males	158 (42.6)
Age (n = 264)	Mean age in years	69
	< 18	15 (5.7)
	18 - 65 years	64 (24.2)
	≥ 65 years	185 (70.1)
CDI classification (n = 374)	HO-HCFA	236 (63.1)
	CA	90 (24.1)
	Unknown	48 (12.8)
Antibiotics exposure within three months before CDI diagnosis (n = 374)	Yes	111 (29.7)
	No	30 (8)
	Unknown	233 (66.3)

HO-HCFA, healthcare facility–onset health-care facility-associated;
CA, community associated.

Regarding previous antibiotherapy, at least 29.7% of the patients received antibiotics within three months before CDI diagnosis, being that 8% had not been exposed to this risk factor. However most of the patients (66.3%) had no data available.

4.3. Antimicrobial susceptibility

The antimicrobial susceptibility for moxifloxacin, vancomycin, metronidazole and rifampicin was investigated for 183 toxigenic strains (Table 4.3).

Overall, 30.1% (55/183) were moxifloxacin-resistant. All the strains from the frequently detected RT027 and RT017 were resistant to moxifloxacin, while for others an important frequency was observed, such as 89% (8/9) for RT126, 50% (6/12) for RT078.

All tested strains were susceptible to vancomycin. When analysing the results for metronidazole it was noticed that only 4 strains were resistant, 3 belonged to RT027 and one to RT651. However, these results need to be confirmed by Etest® strips and/or agar dilution to determine the exact MIC, since the inhibition zone for these isolates was close to the breakpoint cut-off established.

Rifampicin resistance was observed in 21 strains (inhibition diameter = 6 mm), of which 19 belonged to RT017, one to RT043 and one to RT241.

Table 4.3. Antimicrobial susceptibility of *Clostridium difficile* clinical strains, determined by disk diffusion.

Antimicrobial agents	Resistant, no. (%)	Resistance according to RT (no, %)
Moxifloxacin (n = 183)	55 (30.1)	RT027 (18, 100%); RT017 (19, 100%); RT126 (8, 89%); RT078 (6, 50%); RT106 (1, 1.7%); RT241 (1, 50%); RT449 (1, 50%); RTAI-84 (1, 50%)
Vancomycin (n = 183)	0	NA
Metronidazole (n = 183)	4 (2.2)	RT027(3, 17%); RT651 (1,14%)
Rifampicin (n = 183)	21 (11.5)	RT017 (19, 100%); RT043 (1, 50%); RT241 (1, 50%)

NA, not applicable.

Imipenem susceptibility test was performed for 181 strains belonging to 57 different RTs. Only RT017 strains exhibited a resistant phenotype to this antibiotic after 24 h of incubation, with a MIC range between 32 to > 32 mg/L. The strain from RT265, also *tcdA-/tcdB+* was susceptible to imipenem (MIC= 2 mg/L).

Since RT017 has been described as multiresistant, the susceptibility profile for other antibiotics by Etest® was analysed (Table 4.4). All RT017 strains presented high levels of resistance to clindamycin and erythromycin, and were also resistant to tetracycline. On the other hand, strains were highly susceptible to chloramphenicol and tigecycline, with very low MICs.

Table 4.4. Antimicrobial susceptibility of 19 *Clostridium difficile* RT017 strains determined by E-test®.

Antimicrobial agents	MIC range (mg/L) (Etest®)	Resistant, no. (%)
Chloramphenicol	3 - 6	0
Tetracycline	16 – 24	19 (100%)
Tigecycline	0.023 – 0.047	0
Clindamycin	> 256	19 (100%)
Erythromycin	> 256	19 (100%)

4.4. Determinants of antimicrobial resistance

In order to complement the antimicrobial susceptibility data, PCR targeting several determinants of resistance was performed in 338 strains, 308 toxigenic and 30 non-toxigenic. The results for *ermB*, *tetM*, transposons and *catD* are summarized in Table 4.5. The *ermB* gene, conferring resistance to MSL_B antibiotics, was detected in 34 strains (10.1%), while *tetM*, conferring resistance to tetracycline, was identified in 68 strains (20.12%), of which 62 were associated with Tn916-like element (*int*-positive), 4 with a Tn5397 element (*tndX*-positive) and other 4 presented a negative result for both transposons-markers.

Twenty-two strains (6.5%) contained both *ermB* and *tetM* genes and belonged mostly to three toxigenic RTs, RT017, RT126 and RT012, and to one non-toxigenic type, RT039.

Overall, among the 30 non-toxigenic strains tested, 13 (40%) were positive for at least one resistance determinant.

Table 4.5. Distribution of antibiotic resistance determinants according to PCR-ribotype.

No. of strains (n = 338)	*ermB*	*tetM*	*int*	*tndX*	*catD*	RT[a] (no.)
258	-	-	-	-	-	RT027 (40); RT014/0 (27); RT002 (16); RT020 (13); RT005 (7)
12	+	-	-	-	-	RT651 (2); RT012(2); RT009[b] (1); RT010[b] (1); RT031[b] (1); RT039[b] (1); RT106 (1); RT241 (1); RT416 (1); RTAI-83 (1)
42	-	+	+	-	-	RT017 (11); RT078 (15); RT126 (9); RT002 (1); RT005 (1); RT039[b] (2); RT043 (2); RT645 (1)
2			-	+	-	RT012 (1); RT039[b] (1)
2			-	-	-	RT027 (1); RTAI-9-1 (1)
18	+	+	+	-	-	RT017 (8); RT005 (1); RT039[b] (3); RT067[b] (1); RT078 (1); RT106 (1); RT126 (3)
2			-	+	-	RT039[b] (2)
2			-	-	-	RT012 (2)

[a] Mostly representative ribotypes;
[b] Non-toxigenic RT.

All strains were negative for *catD*, which confers resistance to chloramphenicol, and 258 (76.3%) did not carry any of the resistance determinants studied.

The rifampicin-resistant strains were analysed for presence of mutations at *rpoB* gene, using *C. difficile* 630 (CD630_00660) as control. All of the identified amino acid substitutions were located between amino acid 502 and 507 of RpoB. RT017 strains contained both the substitutions His502Asn and Arg505Lys, which were also found in a RT241 resistant-strain. A novel amino acid substitution (Ser507Leu), near the region of mutations associated to rifampicin-resistance, was found in a resistant strain from RT043, which didn't harbour any of the know mutations.

Overall, one substitution in GyrA (Thr82Ile) and one substitution in GyrB (Ser416Ala) were identified in the moxifloxacin-resistant strains analysed. The well-known mutation Thr82Ile was detect in different RTs and was present in of the majority of the moxifloxacin-resistant isolates, including in all RT017 strains, while no mutations were found in GyrB for this RT. RT078 an RT126

moxifloxacin-resistance strains harbour the two mutations, one in GyrA and one in GyrB, although the mutation GyrB (Ser416Ala) seems to not be related to fluoroquinolone resistance, since it has been previously detect in susceptible strains (Spigaglia et al., 2008).

The results of mutations analyses are resumed in Table 4.6.

Table 4.6. Distribution of predicted amino acid substitutions in RpoB, GyrA and GyrB associated with antibiotic resistance according to PCR-ribotypes.

RpoB	GyrA	GyrB	RT (no.)
His502Asn and Arg505Lys	Thr82Ile	none	RT017 (19); RT241 (1)
Ser507Leu	NA	NA	RT043 (1)
NA	Thr82Ile	none	RT027 (18); RT106 (1); RT449 (1); RTAI-84 (1)
NA	Thr82Ile	Ser416Ala	RT078 (6); RT126 (8)

New mutation highlighted in bold face;
NA, not applicable.

Considering the antimicrobial phenotypes and genotypes, the strains from RT017 (*tcdA-/tcdB+*) were the most resistant, presenting simultaneous resistance to several antibiotics representative of six different classes: moxifloxacin (fluoroquinolones), imipenem (carbapenems'), erythromycin (macrolides), tetracycline (tetracyclines), clindamycin (lincosamides) and rifampicin.

For this group of 19 RT017 strains, there was a good correlation between phenotype and genotype for moxifloxacin (*gyrA* mutations), rifampicin (*rpoB* mutations) and tetracycline (presence of *tetM*). Concerning clindamycin and erythromycin, belonging to the MSL_B group, only eight of the 19 strains were *ermB* positive.

Considering that RT017 was one of the most frequently detected RT, was multi-resistant and likely harbours new resistance determinants, few of these isolates were further studied by MLVA and WGS, in order to identify clonal groups and particular genomic features. Since these strains have a particular toxin genotype (*tcdA-/tcdB+*), the only strain from RT256 sharing this genotype was also included in the analysis.

4.5. Study of toxin A-negative/toxin B-positive strains (RT017 and RT265)

The 20 strains with the profile *tcdA-/tcdB+* have only been found in two among the 11 hospital centres: 11 cases from RT017 were detected in a limited period in 2016 (January-July) in hospital A, 1 case from RT265 was isolated in a sample from November 2015, in hospital A, and the remaining 8 were isolated from another hospital (here designated as hospital B), during the study period.

4.5.1. Phylogenetic analysis by multilocus variable number tandem repeat analysis

MLVA was performed for 16 of these strains (15 RT017 and 1 RT265) and compared to the profile of 34 previously isolated strains from RT017, and resulted in 32 unique types (Figure 4.2). Supplementary table 1 contains the MLVA profiles for all the strains.

Concerning RT017 strains, MLVA analysis showed that VNTR *loci* C6 and G8 were the most variable (15-29 and 31-40 repeats, respectively), while the number of repeats in *loci* A6 and E7 was

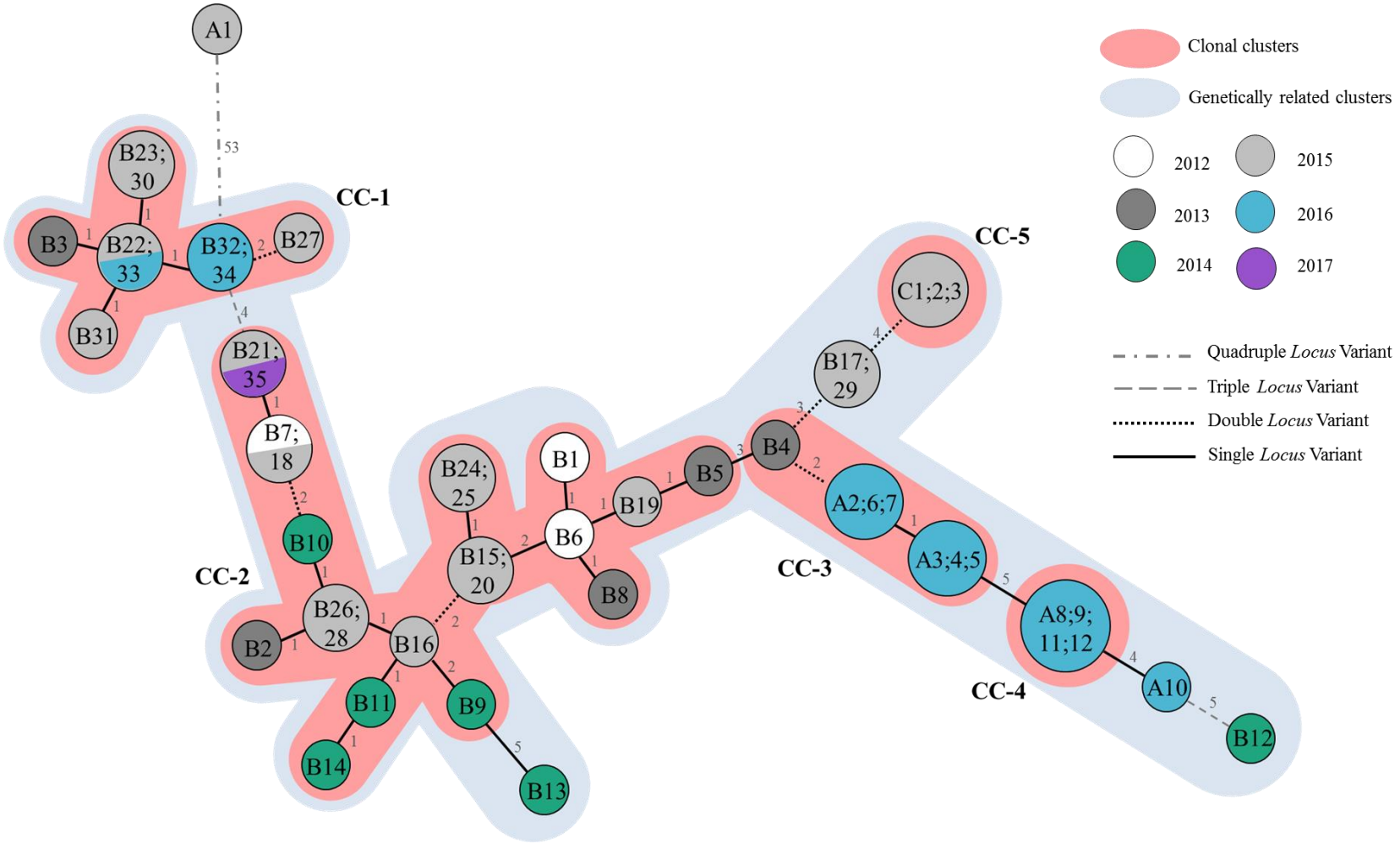

Figure 4.2 Minimum spanning tree of multilocus variable number tandem repeat analysis data for one *Clostridium difficile* RT265 and 49 RT017 isolates. Each circle represents either one unique isolate or more isolates that have identical MLVA types, inside the circles are specified the hospital where the patient was located and the isolates number. The numbers between the circles represent the summed tandem-repeat difference (STRD) between MLVA types. Solid lines represent single *locus* variants, dotted line represent double *locus* variants, dashed line represent 3-*locus* variants and interrupted lines represent 4-*locus* variants between MLVA types. Clonal clusters (CC) are defined by an STRD of ≤ 2, and genetically related clusters are defined by an STRD of ≤10. The isolates are marked in different colors according to the year in which it was isolated as shown in the key (on the right side of the figure). The tree has been redrawn for ease of viewing and is not to scale.

constant or only slightly different (2 and 7-8 repeats, respectively). All the R0T17 strains were genetically related (STDR ≤ 10), comprising 5 clonal clusters (CC).

Isolates from hospital B were collected during six-years (2012-2017), and the respective MLVA types were dispersed by different CC without any link between cluster/year. The largest complex (CC-2) consisted of 21 isolates from hospital B (1 isolate from this study and 20 previous isolates) that were collected during five different years, reflecting the genetic proximity of the isolates, which is consistent with a persistent clone in that hospital setting.

All 11 RT017 isolates from hospital A generated MLVA types that presented single *locus* variation among each other, with an STRD of ≤ 10, indicating that they are highly related to each other, which is consistent with nosocomial transmission. Six of these isolates belong to CC-3, which also includes one isolate from hospital B that was isolated in 2013.

The only isolate from RT265 showed a STRD of 53 from one MLVA type of hospital B isolated in 2016 and belonging to CC-1, with variation in four *loci*, and therefore it is not genetically related to the RT017 isolates.

4.5.1. Whole genome sequencing analysis

WGS was performed for three isolates from hospital A (one from each of the months January, February and July 2016), 5 RT017 strains from hospital B (from August, October and December 2016 and February 2017) and one RT265 strains from hospital A. In addition, WGS data from 25 previously sequenced isolates from RT017 was included in the analysis, 22 from hospital B and three from a third hospital (here designated as hospital C).

In silico MLST analysis of RT017 indicated that all strains belonged to sequence type 37 (MLST clade 4); RT265 assigned as sequence type 88 (MLST clade 4). WGS analysis confirmed the presence of a complete *tcdB* (PubMLST allele 9), the absent of genes coding for CDT and a large deletion in *tcdA*.

A phylogenetic tree was generated based on the core-genome single nucleotide polymorphisms (SNPs) analyses of the RT017 strains, revealing two main groups, one containing the imipenem-resistant strains from hospitals A and B and other single cluster containing three susceptible strains from hospital C (Figure 4.3 A). SNPs analysis showed that all the isolates from hospital A were almost undistinguishable from each other (1 SNPs variant), consistent with nosocomial transmission, while isolates from hospital B were more diverse as they were obtained over several years. Notably, one isolate from 2017 had only 4 SNPs distance from one isolate from 2012, confirming the persistence of the same clones over the years in that hospital. Overall, a similar separation of the isolates by the two analyses can be observed as they apparently distribute themselves in the branches of the SNP-based maximum likelihood phylogenetic tree in the same way that they are distributed in the branches of the MLVA minimum spanning tree. This agreement was firstly observed as the strains of each hospital grouped together but were separated from the other hospitals, being this separation more evident for the SNPs analysis.

All strains revealed a large genome sequence identity, marked by only 74 SNPs when compared to mapping against the *C. difficile* M68 genome (GenBank accession number: NC_017175). Twenty-two of these variant sites discriminate the isolates from this work belonging to hospital A and B, from the previous isolates, from hospitals B and C. Two of these SNPs corresponded to synonymous substitutions, but a considerable number (20) originated, non-synonymous substitutions (Table 4.7). These include genes encoding proteins involved in DNA transcription, cell structure and regulation,

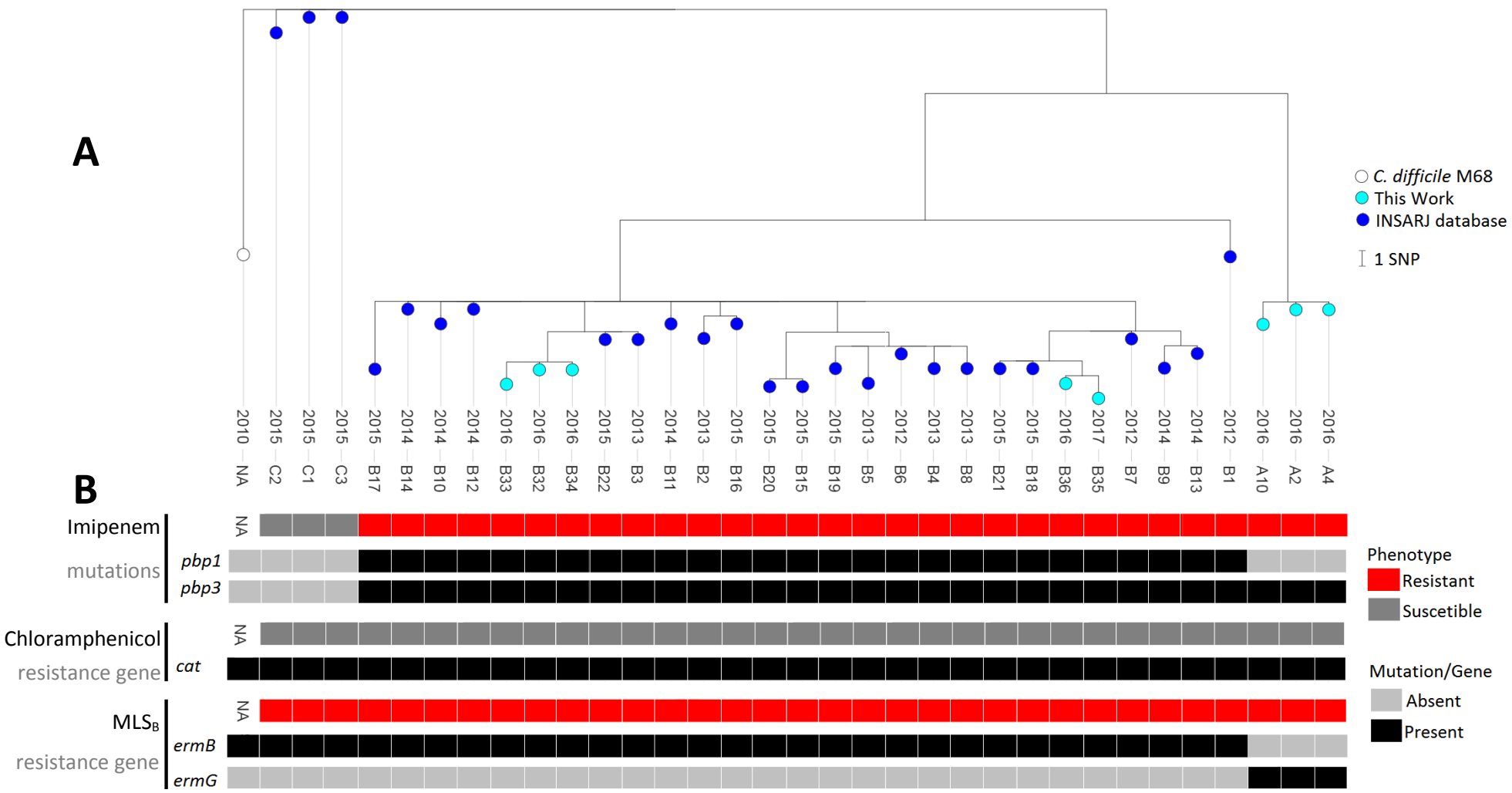

Figure 4.3. Phylogeny of *Clostridium difficile* RT017 clinical isolates and genetic determinants of antibiotic resistance based on WGS sequences. (A) core-genome SNP-based maximum likelihood phylogeny of 33 RT017 *C. difficile* clinical strains generated by mapping reads against to the reference genome *C. difficile* M68. The isolates are marked in different colors according to the work as shown in the key (on the right side of the figure). **(B)** Heatmap visualizing the distribution of antimicrobial resistance determinants together with its associated phenotypes for each isolate (see color key on the right side of the figure). Mutations refer to nucleotide change when compared resistant and susceptible strains; no mutations refer to the absence of nucleotide variations between resistant and susceptible but the genes are present. The capital letter before the isolate number indicates the hospital of origin. A: Hospital A; B: Hospital B, C: Hospital C.

Table 4.7. Unique single nucleotide polymorphisms only found at RT017 strains from this work from RT017 strains previously isolated.

Position [a]	Target *locus* [a]	Nucleotide change [b]	Amino acid change	Product/putative function	Biological Process †
53164	CDM68_RS00405	G605A	Arg202Lys	23S rRNA (guanosine(2251)-2'-O)-methyltransferase RlmB	rRNA methylation
67225	CDM68_RS00470	C2439T	Asp813Asp	DNA-directed RNA polymerase subunit beta'	Transcription
662258	CDM68_RS03290	G266A	Gly89Asp	DNA-binding response regulator	Signal transduction system
902504	CDM68_RS04270	G251T	Gly84Val	TetR family transcriptional regulator	AMR, regulation of transcription
935302	CDM68_RS04430	A820G	Ile274Val	MFS transporter	Transmembrane transport
943701	CDM68_RS04460	A339T	Ala113Ala	ABC transporter ATP-binding protein	AMR, transport
1053823	CDM68_RS04965	A281T	Lys94Ile	hypothetical protein	HP
1392809	CDM68_RS06540	C560T	Ala187Val	translation initiation factor IF-2	Protein biosynthesis
1393468	CDM68_RS06540	G1219A	Ala407Thr	translation initiation factor IF-2	Protein biosynthesis
1553788	CDM68_RS07280	C134T	Thr45Ile	dihydropteroate synthase	Folate biosynthesis
1666351	CDM68_RS07765	G214T	Gly72*	hypothetical protein	HP
1671129	CDM68_RS07795	T209C	Ile70Thr	imidazoleglycerol-phosphate dehydratase	Amino-acid biosynthesis
2010723	CDM68_RS09450	C595A	Pro199Thr	Cell wall-binding repeat-containing protein	Cell structural
2251300	CDM68_RS10605	C407A	Ala136Glu	Lactate utilization protein	Lactate metabolic process
2662461	CDM68_RS12420	G253A	Gly85Ser	amidohydrolase	Proteolysis
3122003	CDM68_RS14375	G1609T	Asp537Tyr	preprotein translocase subunit SecA	Protein transport
3541711	CDM68_RS16075	G700A	Glu234Lys	SAM-dependent DNA methyltransferase	DNA methylation
3641231	CDM68_RS16560	A398G	Asp133Gly	hypothetical protein	HP
3643271	CDM68_RS16570	C775T	Pro259Ser	two-component sensor histidine kinase	Signal transduction system
3795253	CDM68_RS17445	C30A	Cys10*	HxlR family transcriptional regulator	Transcription
4026545	CDM68_RS18530	C220T	Gln74*	SAM-dependent methyltransferase	DNA methylation
4045861	CDM68_RS18610	C578T	Ala193Val	hypothetical protein	HP

[a] Genome position relative to the annotation of the *C. difficile* M68 genome (accession number NC_017175);
[b] The nucleotide changes are presented in the 5' to 3' direction and is relative the reference *C. difficile* M68 genome;
† according to http://www.ebi.ac.uk/interpro
Nonsense mutations leading to putative protein truncation are represented by an asterisk (*);
AMR, antimicrobial resistance; HP, hypothetical protein.

response to environmental changes and metabolic reactions. It also includes hypothetical proteins and possible genes implicated in antimicrobial resistance as well. Nonsense mutations resulting in premature truncation of the encoding protein was observed in genes encoding for a S-adenosyl-methionine (SAM)-dependent methyltransferase, for a transcriptional regulator (HxlR family transcriptional regulator) and for a hypothetical protein.

C. difficile usually contains four HMW PBP, except the strains from RT017, which contain a fifth *pbp* gene, here designated as *pbp5* (corresponding to CDM68_RS02615 *locus* tag in *C. difficile* M68 strain). This gene was also found in the RT265 strain, in the same genomic context as the RT017 strains, with both PBP5 sharing 99% homology.

All the imipenem-resistant strains, from hospitals A and B, harboured mutations in HMW PBP genes, located near conserved motifs, when compared to imipenem-susceptible strains, but notably with different profiles: the resistant strains from hospital B have the two mutations previously reported (Isidro et al. 2017), one (G1663A) in *pbp1* (corresponding to CDM68_RS04280 *locus*) leading to an amino acid substitution Ala555Thr, and the other (A2162C) in *pbp3* (corresponding to CDM68_RS05670 *locus*) resulting in the amino acid substitution Tyr721Ser; isolates from hospital A harbour only the mutation (A2162C) in *pbp3* (Figure 4.3 B). For this clone, no mutations were found in other PBPs encoding genes (*pbp1*, *pbp2*, *pbp4* and *pbp5*).

In silico screening of antimicrobial resistance genes based on WGS analysis using CARD and ResFinder databases, revealed the presence of several genes in this category. First, a gene encoding a putative chloramphenicol acetyltransferase CAT (CDM68_RS02605) was found in all the strains, although they were susceptible (Figure 4.3 B).

The isolates from hospital B were positive for *ermB* gene, previously associated with resistance to MLS_B antibiotics in *C. difficile*, confirming the results of the PCR screening, while all strains from RT017 from hospital A were positive for *ermG* gene. Both genes encode a rRNA adenine N-6-methyltransferase. Homology search of *ermG* using nucleotide Blast showed that this gene shared 100% identity to *ermG* gene found in several other Gram-positive species, such as *Enterococcus cecorum* (RA45_RS03490 *locus*) and *Lysinibacillus sphaericus* (A7J11_00653 *locus*). Transformation of *C. difficile* strain 630Δ*erm* with a plasmid containing *ermG* resulted in a resistance phenotype for clindamycin and erythromycin, with MIC > 256 mg/L, showing that this gene is functional in *C. difficile* and is associated with high level of resistance to both these antibiotics.

The *ermG* gene was located inside a large region of 63.4 kb that displays traces of horizontal gene transfer (encompassing recombinases, phage-related proteins and flanked by transposases), as shown in Figure 4.4. Nucleotide Blast was conduct for the entire region, showing high degree of homology with an identical region present in a *C. difficile* non-toxigenic strain (*Peptoclostridium difficile* strain Z31, GenBank accession number: CP013196.1). Table 4.8 lists the suggested annotation and homologues *locus* tag (PubMed) for all the ORFs found at this putative mobile element containing the *ermG* gene. A streptogramin A acetyltransferase was also detected in this putative mobile element.

Screening for resistance determinants at ResFinder also showed homology to other two genes possible involved in MLS_B resistance, the *mefA* and the *msrD* genes, located near the *ermG* on the putative mobile element (Figure 4.4); blast of the nucleotide sequence gave 96% for a *mefA* gene (A6J31_02365) and 98% of identity for *msrD* (A6J31_02370) in *Streptococcus sp*. Those genes encode for a macrolide efflux MFS transporter Mef(A) and a ABC-F type ribosomal protection protein Msr(D), respectively (Table 4.8).

Regarding the other classes of antibiotics, WGS analysis confirmed the results of the PCR screening.

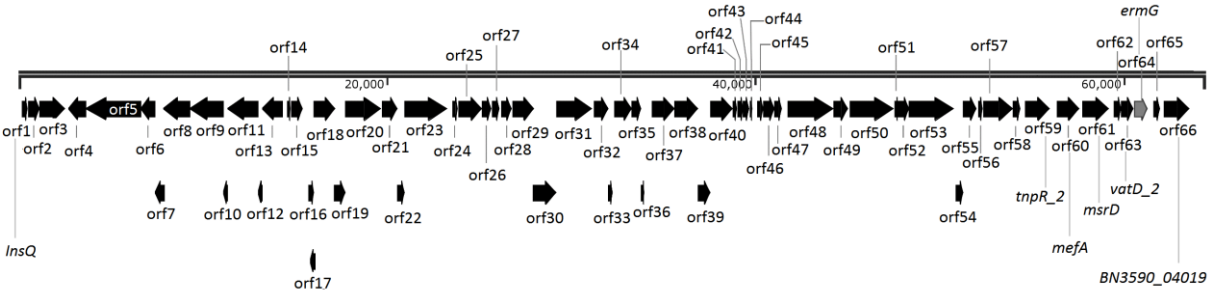

Figure 4.4. Genetic organization of the putative transposon in *Clostridium difficile*. Schematic representation of the putative mobile-element (63,400 bp) harboring the *ermG* gene in the genome of *C. difficile* MLS_B-resistant strains; suggested gene annotation based on Nucleotide BLAST. Arrows indicate open reading frames (ORFs) and direction of transcription, size is proportional to gene length; *ermG* is shown in grey, with the remaining ORFs shown in black.

For the isolate from RT265 no resistance determinant was found, in accordance to its antibiotic susceptibility profile.

Table 4.8. Open reading frames and putative proteins that compose the mobile element carrying the *ermG* gene.

ORF	GenBank *locus* tag	Product
orf1	CDM68_RS02180	transposase
orf2	CDM68_RS02185	RNA pseudouridine synthase
orf3	CDM68_RS02190	23S rRNA (uracil(1939)-C(5))-methyltransferase RlmD
orf4	Thet_1692	methionine sulfoxide reductase A
orf5	PCZ31_3874	Type-1 restriction enzyme R protein
orf6	PCZ31_3873	hypothetical protein
orf7	PCZ31_3872	hypothetical protein
orf8	PCZ31_3871	EcoKI restriction-modification system protein HsdS
orf9	PCZ31_3870	putative type I restriction enzymeP M protein
orf10	PCZ31_3869	helix-turn-helix protein
orf11	PCZ31_3868	hypothetical protein
orf12	PCZ31_3867	hypothetical protein
orf13	PCZ31_3866	hypothetical protein
orf14	PCZ31_3865	hypothetical protein
orf15	CDM120_RS02285	sigma-70 family RNA polymerase sigma factor
orf16	PCZ31_3863	hypothetical protein
orf17	Thet_1678	RNA biogenesis protein rrp5, putative
orf18	PCZ31_3861	PD-(D/E)XK nuclease superfamily protein
orf19	CDM120_RS02305	DUF2815 domain-containing protein
orf20	PCZ31_3859	DNA polymerase I, thermostable
orf21	PCZ31_3858	Phage antirepressor protein KilAC domain protein
orf22	PCZ31_3857	hypothetical protein
orf23	PCZ31_3856	Virulence-associated protein E
orf24	PCZ31_3855	VRR-NUC domain protein
orf25	PCZ31_3854	RNA polymerase-associated protein RapA

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ORF	GenBank *locus* tag	Product
orf26	CDM120_RS02340	phage-associated protein
orf27	PCZ31_3852	HNH endonuclease
orf28	PCZ31_3851	Phage terminase, small subunit
orf29	PCZ31_3850	S-adenosylmethionine synthase
orf30	PCZ31_3849	DNA adenine methyltransferase YhdJ
orf31	PCZ31_3848	putative BsuMI modification methylase subunit
orf32	PCZ31_3847	hypothetical protein
orf33	PCZ31_3846	hypothetical protein
orf34	CDM120_RS02375	amidoligase
orf35	PCZ31_3844	AIG2-like family protein
orf36	PCZ31_3843	hypothetical protein
orf37	PCZ31_3842	Phage Terminase
orf38	PCZ31_3841	Phage portal protein
orf39	PCZ31_3840	TP-dependent Clp protease proteolytic subunit
orf40	PCZ31_3839	Phage capsid family protein
orf41	PCZ31_3838	hypothetical protein
orf42	PCZ31_3837	Phage gp6-like head-tail connector protein
orf43	PCZ31_3836	Phage head-tail joining protein
orf44	PCZ31_3835	hypothetical protein
orf45	PCZ31_3834	hypothetical protein
orf46	CDM120_RS02440	phage major tail, phi13 family protein
orf47	PCZ31_3832	hypothetical protein
orf48	PCZ31_3831	hypothetical protein
orf49	PCZ31_3830	Phage tail protein
orf50	PCZ31_3829	Prophage endopeptidase tail
orf51	PCZ31_3828	hypothetical protein
orf52	PCZ31_3827	hypothetical protein
orf53	PCZ31_3826	Glycosyl hydrolases family 18
orf54	PCZ31_3825	Holin family protein
orf55	PCZ31_3824	N-acetyl-anhydromuranmyl-L-alanine amidase
orf56	PCZ31_3823	hypothetical protein
orf57	PCZ31_3822	Transposon gamma-delta resolvase
orf58	CM240_1374	recombinase
orf59	PCZ31_3820	Transposon gamma-delta resolvase
orf60	A6J31_02365	macrolide efflux MFS transporter Mef(A)
orf61	A6J31_02370	ABC-F type ribosomal protection protein Msr(D)
orf62	PCZ31_3817	dephospho-CoA kinase/protein folding accessory domain-containing protein
orf63	PCZ31_3816	Streptogramin A acetyltransferase
orf64	RA45_RS03490	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(G)
orf65	PCZ31_3814	IS66 Orf2 like protein
orf66	PCZ31_3813	Transposase C of IS166 homeodomain protein

Suggested annotation based on Nucleotide BLAST.

5. Discussion

Although CDI represents a great healthcare burden in developed countries, data in Portugal is still scarce with only a few reported studies. The present study provides an overview of the current state of *C. difficile* strains circulating in some hospitals in Portugal, concerning prevalent RTs and antimicrobial resistance rates and its associated resistance mechanisms, focusing on the *tcdA-/tcdB+* and its molecular epidemiology. The results presented herein only reflects the distribution of strains in public hospitals that voluntarily agreed to contribute to the laboratory surveillance of CDI, and therefore it is not representative of the country overall.

Of the 455 samples positive for *C. difficile* by EIA tests, 378 were recovered by culture, which is the golden standard method (Crobach et al., 2016). Therefore, the 77 samples with a negative culture may represent false positive diagnostics performed in the hospitals.

The majority of the patients (70.1%) were 65 years or older, which is consistent with the criteria for disease severity defined by the ESCMID guidelines (Debast et al., 2014). A total of 5.7% were collected from children, suggesting that the epidemiology in the country is changing.

It was not possible to gather detailed epidemiological data for all the patients included in the study. For example, it could not be determined the antibiotic prescription data for 66.3% of the individual patients, making difficult to interpret the meaning of this factor in the onset of CDI; or either information about previously patient's hospitalizations to distinguish whether infections were community or hospital acquired due to recent hospital contact. As a result, some of the cases attributed as community-associated could have a hospital background that could change the analyses. Even so, most of the cases were defined as healthcare facility–onset, health-care facility-associated (63.1%) which is consistent with previously *C. difficile* epidemiology in the country (Santos et al., 2016).

Concerning distribution of RTs, RT027 still remains the most frequently found, in agreement with a surveillance study conducted between 2010 and 2015 (Santos et al., 2016). RT027 was also reported as the most common RT in a European multicentre study of CDI in hospitalised patients with diarrhoea, including 19 countries, carried out between 2012 and 2013 (Davies et al., 2016), with variable frequencies between countries. In the present study, RT014 was the second most frequently as in the previous Portuguese study and also in agreement with the European study. Although here RT106 was the third most representative RT, it was not detected among the ten most commonly isolated RTs in the European multicentre study nor in the study performed before in Portugal before (Davies et al., 2016; Santos et al., 2016). This RT106 can be considered as an emergent RT in English hospitals, where it was found with high resistance rates to erythromycin and moxifloxacin (Brazier et al., 2008; Sundram et al., 2009); being also found in companion animals in different geographic locations (Álvarez-Pérez et al., 2017; Silva, Rupnik, Diniz, Vilela, & Lobato, 2015). Therefore, it is important to keep investigating the molecular epidemiology of the organism locally.

The European multicentre study also established a relationship between RTs diversity and prevalence of RT027, where diversity decreased as the prevalence of RT027 increased (Davies et al., 2016), suggesting that countries with a high frequency of RT027 strains would have an overall lower RT diversity. But this does not seem to be the current situation for Portugal, as in this study a great diversity of RTs were found (75 RTs including RT027), these results could be due to the collaboration of a higher number of hospitals, allowing a better view of the high variability in the country. Nevertheless, this RT diversity (namely within hospitals), also makes tempting to suggest that other transmission routes, in addition to contact between individuals in hospital-setting, are involved in the CDI cases. It should be noted that *C. difficile* epidemiology is also spreading to food animals on farms and companion animals that contact with an amount of people (Álvarez-Pérez et al., 2017; Knight et al.,

2017; Thitaram et al., 2016), in river water and soil too (Janezic et al., 2012). *C. difficile* strains have also been shown to persist in the environment for a number of years (Cairns et al., 2015). Therefore, studies to investigate the RT prevalence in animals and the potential role of surface contamination in hospitals in the spread of *C. difficile* strains locally could be useful to understand this high variability.

The pattern of antimicrobial resistance seems to differ among different RTs. Fluoroquinolone resistance has been reported worldwide; in a recent study from U.S. (Kociolek et al., 2016) a considerable proportion of moxifloxacin-resistant strains was found (36%), being related to the significantly higher proportion of RT027 strains among those patients. A pan-European longitudinal surveillance of antibiotic resistance among prevalent *C. difficile* RTs (Freeman et al., 2015) also reported variable rates of resistance between countries, overall 40% of the analysed strains were moxifloxacin-resistance, being mainly associated with specific and common RTs found in the study, such as RT356, RT018, RT027, and RT017. In the present work, resistance to moxifloxacin was observed in a frequency of 30.1% and was mainly associated with RT027 and RT017, which is not different from a previous study in Portugal (Santos et al., 2016). Those were also the most frequent RTs which may indicate a selective advantage for resistance to fluoroquinolone agents. Moxifloxacin-resistance was also found for other less frequent but highly virulent (CDT positive) RTs at higher proportions, such as RT126 (89% of the tested strains) and RT078 (50% of the tested strains). In contrast, in the previous Portuguese study, moxifloxacin resistance was not a common feature of RT078 isolates, and they were reported only in the last 2 years of the study (2014 and 2015) (Santos et al., 2016); in an Irish study, only 27.5% of the clinical isolates from RT078 were resistant to fluoroquinolones (Solomon et al., 2011). Results for RT126 is in agreement with an Italian study where the majority of the fluoroquinolone-resistant strains analysed belonged to RT126 (Spigaglia, Barbanti, Dionisi, & Mastrantonio, 2010). Overall these results show that emergency of fluoroquinolone-resistant strains is dependent on the country, and suggest that an evolution towards the dissemination of more resistant strains in ongoing. All resistant strains were tested for point mutations in the QRDR, of GyrA and a substitution Thr82Ile was found in all resistant isolates, while only one GyrB substitution (Ser416Ala) was found in one strain. However, this GyrB substitution is not associated with fluoroquinolone resistance, since it had already been observed in susceptible strains (Spigaglia et al., 2008).

There was no evidence for vancomycin resistance in our study. Decreased susceptibility for this antibiotic has been reported, but with reduced rates. In the pan-European study almost every strain was susceptible to vancomycin (96.84%) (Freeman et al., 2015); in a recently Portuguese study, two isolates had decreased susceptibility to vancomycin (0.4%) belonging to RT001 and RTAI-58 (Santos et al., 2016). Therefore, this antibiotic remains the preferred treatment option for treating patients suffering from moderate to severe CDI and for those that do not respond to initial therapy with metronidazole (Debast et al., 2014). For metronidazole, only four strains were classified as resistant, three of them belong to RT027. Strains from this RT were also found to be resistant to metronidazole in the previous Portuguese survey (Santos et al. 2016) and among the 2.2% European metronidazole-resistant strains (Freeman et al., 2015). Notably, one of the patients harbouring the metronidazole-resistant strain was previously exposed to this antibiotic. Although no information was available for the others patients, with the usage of metronidazole for CDI treatment it is possible that they had been exposed to this antibiotic, as such, this phenotype could probably due to the selective pressure of the antibiotic usage. Moreover, because the susceptibility test was conducted by disk diffusion, it's possible that the resistance rates were overestimated as some inhibition zone near the breakpoint may be inaccurately determined. Besides that, those putative resistant strains should be confirmed using the gradient diffusion by Etest® strips or even by agar dilution test as recommended by EUCAST guidelines (v.6.0; http://www.eucast.org), to confirm these results and get the exact MIC.

Rifamycin's resistance was reported in a United States study with rates of 1.6% for paediatric isolates and 6.7% for adults isolates (Kociolek et al., 2016). In the pan-European study resistance frequency for rifampicin was 13.4%, however, with variable rates between countries (from 0 to 62.4%) (Freeman et al., 2015). In the current study, 11.5% of the tested strains were rifampicin-resistance, although lower than the overall European rate, was higher to those observed in the majority of the countries. Rifampicin resistance was detected in all RT017 isolates, as well as in a RT241 (1/2, 50%): two already know amino acid substitutions in RpoB were detect, His502Asn and Arg505Lys (Curry et al., 2009). However, a novel amino acid substitution (Ser507Leu) was observed in the only RT043 resistant-strain found, which didn't harbour any of the know mutations. Being therefore likely associated with this phenotype. Previous studies with *Staphylococcus aureus* and *Mycobacterium tuberculosis* have shown that independently derived mutations in these residues are frequently detected, being that an equivalent Serine to Leucine substitution was already describe for *S. aureus* resistant strains (Murphy et al., 2006; O'Connor et al., 2008). Resistance to rifampicin may compromise the use of rifaximin, considered an alternative therapy for relapsing CDI. (Curry et al., 2009; Debast et al., 2014).

Imipenem resistance is not well documented in *C. difficile*. In the present study only RT017 strains were resistant to imipenem. A Korean study also reported resistant for this antibiotic in RT017 (12%), simultaneously with the introduction of the drug at the hospitals (Lee, Lee, Lee, Riley, & Kim, 2014). In the pan-European study it was reported a 7.4% imipenem-resistance rate, but no distribution per RTs was described. Nonetheless, the geometric mean imipenem MICs were highest among RT027 and RT106.

In the present study, the distribution of resistance determinants for MSL_B antibiotics (*ermB* gene) and for tetracycline (*tetM* gene) were highly variable between and within RTs. Overall, *ermB* gene was detected in only 10.1% of the strains tested; in a study conducted by Wasels, Spigaglia, Barbanti and Mastrantonio (2013) they reported a burden associated with the *in vitro* fitness in strains harbouring mobile elements containing the *ermB*-gene. This could be the explanation behind the low frequency of this gene in our study.

Concerning the *tetM* gene, used to estimate tetracycline resistance strains, a low rate was observed (20.1%) as compared to a study conducted in China where *tetM* frequency reached 41.7% in RTs such as RT017, RT065, RT014, RT046 and RT002 (Dong et al. 2013). In our study, *tetM* was ubiquitous among RT017, RT126 and RT078, showing that it is associated with specific RTs, which may explain differences among studies. Most of the strains harboured the Tn916 transposon while a small fraction carried the Tn5397 element which is in agreement with other reports (Dong et al., 2013; Spigaglia et al., 2005). Of relevance, four *tetM*-positive strains were negative for those two transposons markers. Further studies should be carried on these strains using WGS in order to characterize putative novel *tetM*-carrying elements. Forty percent of the non-toxigenic strains tested harboured at least one resistance determinant (*ermB* or *tetM*), as such, it should be noted that these generally unnoticed strains could act as a pool of resistance determinants transmissible to toxigenic strains.

The *catD* gene, which confers resistance to chloramphenicol, was not found in any of the strains tested; despite the small sample (n = 338), it can be speculated that this gene does not circulate in Portugal. The causes of the relatively low incidence of chloramphenicol-resistant *C. difficile* in the country are not known, although it's conceivable that would be related to the local drug prescription habits. Accordingly, in the pan-European study, most prevalent RTs were largely susceptible (95–100% of isolates) to chloramphenicol. Clusters of chloramphenicol-resistant isolates were found only in Germany, The Netherlands and Latvia (Freeman et al., 2015).

Aside from an European study were a set of multidrug resistant isolates were characterized (particularly belonging to RT001, RT012 and RT017) (Spigaglia et al., 2011), in the current study, the

most prominent multidrug resistant RT was RT017. Only one strain belonging to RT241 was also associated with multidrug resistance, harbouring the *ermB* resistance gene and mutations conferring resistance to moxifloxacin and rifampicin. In addition, when tested for further antibiotics, all RT017 strains showed simultaneous resistance to six different classes of antibiotics. According to Coia (2009) the use of a particular antimicrobial agent could result in selective advantage for the spread of *C. difficile* if the organism is resistant to that particular drug. Therefore, it is tempting to suggest that multidrug resistance would be a good driver of CDI epidemics. Having that in mind, in the currently study, three different techniques (phenotypic characterization, MLVA and WGS) were used to characterize toxin A-negative/toxin-B-positive group of strains to which the multidrug resistant RT017 belongs.

Twenty strains were *tcdA-/tcdB+* of which, 19 belonging to RT017 and one to RT265. To date, there were no reports of RT265 strains in Portugal. According to van Dorp et al. (2017) this RT appears to be very uncommon or absent in North American and European countries, except for Belgium and the Netherlands being primarily isolated from children aged < 2 years. In our study, this RT was isolated in an elderly patient. WGS analysis showed that RT265 shares many features with RT017, such as: an intact *tcdB* gene, a 1.8 kb deletion in *tcdA*, and no genes of CDT genes, according to previous data (van Dorp et al., 2017). This strain also belonged to MLST clade 4 and was assigned toST88. Also, similarly to RT017, this RT contains a gene coding for a fifth HMW PBP. Overall, these indicates that these two RTs are close, although belong to different clusters by MLVA and SNPs analysis.

Regarding *C. difficile* RT017, despite the loss of the capability to produce both toxins, this RT is increasingly becoming a potential epidemic strain, being isolated over the past decade in Asian as well as emerging in a few European countries (Arvand et al., 2009; Dobreva et al., 2013; Lee et al., 2014; Yang et al., 2017). In the currently study, RT017 make up 5.1% of the isolates, which is pretty much similar to the five years study previously conduct in Portugal (Santos et al., 2016). It is worth noting that all the isolates found are either endemic, such as the ones found in hospital B, or associated with an outbreak, such as in hospital A (since all the isolates from that hospital remote from a short period of time, sharing the same molecular characteristics), with no single cases found, showing the virulence and epidemics of these strains.

As already mentioned above, resistance was found for moxifloxacin, rifampicin and imipenem, plus the presence of *tetM* gene for all the strains and *ermB* in hospital B isolates. Those RT017 strains were also tested for antibiotic susceptibility for other drugs; as result, all of the isolates in the present study were susceptible to chloramphenicol and tigecycline and resistant to tetracycline. Interestingly the presence of *tetM* gene in all strains seems to have an effect on tetracycline susceptibility but not to tigecycline (a tetracycline derivative antibiotic), probably because this third generation drug display enhanced antimicrobial activity overcoming ribosome protection mechanisms as the one promoted by *tetM* (Dönhöfer et al., 2012).

All RT017 isolates also had high-level erythromycin and clindamycin-resistance, including the hospital A strains classified as *ermB*-negative; for those strains the *ermG* gene was found by WGS analyses. This e*rmG* gene encodes for an rRNA adenine N-6-methyltransferase, which can methylate adenine at position 2058 of 23S rRNA, conferring resistance to MLS_B antibiotics. This gene has appeared in human colonic *Bacteroides* species where it was found on conjugative transposons (Shoemaker, Vlamakis, Hayes, & Salyers, 2001; Wang et al., 2003). Although, the results presented here do not prove that DNA was transferred directly from these species to *C. difficile,* the data indicates that this gene was transferred by a mobile element; Wasels et al. (2014) have already provides evidence of the possible transmission of antibiotic resistance determinants among pathogenic bacteria occupying the same human intestinal niche, which may have happened in that case. In *C. difficile* the presence of

ermG gene was previously reported in one non-toxigenic strains (RT009), but without any phenotype associated (Pereira et al., 2016).

The *ermG* gene was shown to be associated with high level of resistance to clindamycin and erythromycin, through transformation of *C. difficile* 630Δ*erm* with a plasmid containing *ermG*. Despite its previous occurrence in a non-toxigenic strain, this is the first time it is described in toxigenic strains, and associated with a resistance phenotype. Its presence only in the strains belonging to hospital A may be indicative of recent gene transfer, highlighting the importance of an active surveillance of resistance determinants.

Since *ermG* is located in a mobile element, there is the possibility to spread via horizontal gene transfer. The *ermB*-negativity in high-level MSL_B-resistant isolates is a well-known phenomenon (Nyc et al., 2016; Spigaglia et al., 2011), and therefore it can be speculated if some of these reported strains harboured the *ermG* gene. Further studies should be also carried to understand if the carriage of the element containing the gene would have fitness cost associated.

The presence of *mefA* and the *msrD* genes were also observed in this mobile element; those two genes are known to promote low or moderate levels to macrolide resistance by active efflux of the antibiotic, due to an efflux pump that belongs to the major facilitator superfamily encoded by the *mefA* gene, or by an efflux protein belonging to the ABC transporter superfamily encoded by *msrD*. The *msr* group differs from the *mef* genes because it confer resistance to both macrolide and streptogramin B antibiotics, although for both, clindamycin is neither an inducer or a substrate for the pump, and thus the strains caring those genes are fully susceptible to this antibiotic (Leclercq, 2002; Metcalf et al., 2017). Since the RT017 strains had high-level resistance to the later antibiotic, we confirm that these genes don't have a role in resistance. In addition, the presence of *mefA* and the *msrD* genes could only explain low or moderate resistance levels to macrolides or streptogramin B, again emphasizing the role of *ermG*.

These results indicate that our real MLS_B-resistance rate must be higher than the one deduced from the frequency of the *ermB* gene, since other mechanisms can be involved; therefore, all strains should be tested for antibiotic susceptibility to identify the resistant strains, and then the associated genetic determinants should be characterized. As reported by the European Centre for Disease Prevention and Control (2016), MLS_B antibiotics are one of the most common drugs used in both community and hospital sector in Europe, what makes really important to keep track on the resistance patterns for these group of antibiotics.

The putative mobile element also contained a gene encoding a streptogramin A acetyltransferase. This protein belongs to the large family of virginiamycin O-acetyltransferase (Vat) enzymes, known to cause resistance to the streptogramin A antibiotics (Stogios et al., 2014), however this group was not tested here, hence, its potential impact in antibiotic susceptibility of those *C. difficile* strains is unknown.

Concerning imipenem resistance, mutations in HMW PBP genes were found in all RT017 resistant strains, as in an earlier study where RT017 collected between 2012–2015 carried mutations in PBPs and showed statistically higher imipenem MIC than the isolates with no mutations (Isidro et al., 2017). In that study two mutations (at *pbp1* and *pbp3*) were thought to be causative of resistance, although, in the current study all the resistant strains from hospital A harboured only mutations in *pbp3* (Figure 3.3 B). So, it's legitimate to speculate if this single mutation is the one crucial for resistance. Yet those results show clearly that the interpretation of such data is not going to be straightforward, and more studies need to be done. Portugal has been one the major consumers of carbapenems in the hospital sector in Europe (European Centre for Disease Prevention and Control, 2016), as Dingle et al. (2017)

associated RT027 fluoroquinolone-resistant emergency with fluoroquinolone use, it's conceivable to conclude that the emergence of carbapenems resistance in *C. difficile* is likely due to the high usage of this class of antibiotic in the recent years, highlighting the importance of a responsible use of antibiotics.

MLVA was performed for RT265 and RT017 strains. Minimum spanning tree analysis clearly demonstrate the genetic relationships among the RT017 Portuguese isolates being possible to identify five clonal clusters. Furthermore, the fact that a group of isolates were collected from the same hospital within a 6-years period, suggests that this degree of similarity among MLVA types is highly indicative of nosocomial transmission and persistence of the same clones over the years.

The VNTR *loci* used in this study were previously demonstrated to be useful for epidemiologic investigation of *C. difficile* outbreaks and nosocomial transmission (Marsh et al., 2006); their utility for detection of outbreaks was demonstrated by analysis of a group of RT017 from hospital A which clustered on the same branch of the minimum-spanning tree indicating a genetic relatedness among these isolates.

Previous studies using MLVA have demonstrated genetic relatedness among a large collection of *tcdA*-/*tcdB*+ strains from seven countries (van den Berg et al., 2004). In this study, RT265 revel to be distance from the others analysed strains, consistence with the fact it belongs to a different RT.

In summary, the MLVA results indicate that INSARJ's isolates collection consists primarily of three major complexes of highly related isolates, suggestive of hospital-acquired infections.

A phylogenetic tree of SNPs identified through WGS analyse revealed two main related groups comprising 33 RT017 clinical isolates that distinguish the imipenem-resistant strains from the susceptible ones. Nevertheless, it was possible to distinguish the local RT017 isolates from the international control strain (*C. difficile* M68). Overall, 74 SNPs were found which includes variants between the clinical strains and the reference in sequences that have an ortholog to a known gene sequences, and 22 of these variant sites were able to discriminate the isolates from this work belonging to hospitals A and B, from the previous isolates, collected from hospitals B and C.

Among this 22 SNPs variations there was a series of non-synonymous mutations in important genes, including genes involved in the metabolism and antibiotic resistance, mutations leading to truncated proteins were observed for a hypothetical protein, a transcriptional regulator and SAM-dependent methyltransferase, the last one associated to diverse metabolic functions in biocatalysis and biosynthesis (Struck, Thompson, Wong, & Micklefield, 2012); the meaning of these findings has to be studied in future works. The high number of SNPs found in hypothetical proteins is also very interesting, and this group should be further studied to predict their function and the effect of the point-mutations found in some of them.

Remarkably, the most recently strain and one of the most ancient strain from hospital B, separated by a period of 6-years, had only 4 SNPs distance, what is consistent with the evolutionary mutation rate estimated by Eyre et al. (2013) of 0.74 SNPs per year, suggesting that the isolates share a common ancestor. This result supports the idea that a clonal strain from RT017 is endemic in hospital B, persisting for at least 6-years leading to nosocomial transmission. Further studies are needed to investigate the persistence of these strains in the hospital setting.

WGS results also suggest that the clonal strains persisting in hospital B is different from the hospital A strains. Additionally, all the isolates from hospital A were tight closely, reinforcing that an outbreak has occurred. How this outbreak clonal strain was inserted in the hospital remains unclear, but it could be due a patient that was infected in the community and spread the strain during its hospital

stay, which raises many concerns especially on the spread of this multidrug resistant strain in the community.

In conclusion, the current work gives a contribution to the knowledge of the molecular epidemiology and resistance patterns of *C. difficile* in Portugal, especially of the emergent strains of RT017, which are multiresistant and associated with endemic or epidemic cases of CDI. A new resistance determinant, found in a mobile element, was characterized for the first time in *C. difficile* toxigenic strains. The results also suggest that the lineage of strains from RT017 appears to be constantly evolving, acquiring new resistance determinants, which highlights the need for continued epidemiological and antimicrobial surveillance. Finally, this work provides the foundation for future studies, namely the role in RT017 evolution of SNPs found in several proteins, including many hypothetical ones; to study the spread and impact on the fitness of the new mobile element, as well as to assess whether this element can be easily transferred among clinical strains, from the same or different RTs, by horizontal gene transfer.

6. References

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7. Supplementary information

Supplementary table 1. Multilocus variable number tandem repeat analysis profiles for *Clostridium difficile tcdA-/tcdB+* isolates.

Isolate / *Loci*	A6Cd	B7Cd	C6Cd	E7Cd	G8Cd	CDR60
A1	29	15	27	10	18	10
A2	2	11	20	8	32	8
A3	2	11	21	8	32	8
A4	2	11	21	8	32	8
A5	2	11	21	8	32	8
A6	2	11	20	8	32	8
A7	2	11	20	8	32	8
A8	2	11	15	8	32	8
A9	2	11	15	8	32	8
A10	2	11	15	8	36	8
A11	2	11	15	8	32	8
A12	2	11	15	8	32	8
B1	2	8	21	8	36	7
B2	2	8	24	8	35	8
B3	2	9	28	8	33	8
B4	2	10	21	8	32	7
B5	2	10	21	8	35	7
B6	2	9	21	8	36	7
B7	2	10	24	8	34	7
B8	2	9	21	8	35	7
B9	2	10	23	8	35	7
B10	2	8	24	8	34	7
B11	2	8	23	8	34	7
B12	2	8	15	8	35	7
B13	2	10	23	8	40	7
B14	2	8	23	8	33	7
B15	2	9	23	8	36	7
B16	2	8	23	8	35	7
B17	2	8	21	8	31	7
B18	2	10	24	8	34	7
B19	2	10	21	8	36	7
B20	2	9	23	8	36	7
B21	2	10	25	8	34	7
B22	2	9	28	8	33	7
B23	2	9	28	8	34	7
B24	2	9	24	8	36	7
B25	2	9	24	8	36	7
B26	2	8	24	8	35	7
B27	2	9	27	7	34	7
B28	2	8	24	8	35	7

Supplementary table 1. continued page 2 of 2

Isolate / *Loci*	**A6Cd**	**B7Cd**	**C6Cd**	**E7Cd**	**G8Cd**	**CDR60**
B29	2	8	21	8	31	7
B30	2	9	28	8	34	7
B31	2	9	29	8	33	7
B32	2	9	27	8	33	7
B33	2	9	28	8	33	7
B34	2	9	27	8	33	7
B35	2	10	25	8	34	7
C1	2	8	18	8	3	6
C2	2	8	18	8	31	6
C3	2	8	18	8	31	6